DRAFT DETAILED REVIEW PAPER

ON

MYSID LIFE CYCLE TOXICITY TEST

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TABLE OF CONTENTS

2

3

List of Tables

List of Figures

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1.0 EXECUTIVE SUMMARY

Endocrine disruptors are any chemicals that are known or suspected to cause adverse endocrine effects in organisms or their progeny. Such chemicals have received increased attention over the past decade because of the potential harm they can do to wild and domestic animals and ultimately to humans. Therefore, Congress authorized the United States Environmental Protection Agency (EPA) to develop a program to screen a wide array of chemicals found in drinking-water sources and food to determine whether they possess estrogenic or other endocrine activity that could have disruptive endocrine effects in humans. The aim of this program is to develop a two-tiered approach: that is, a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1), and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. The organisms used in the screening and testing will represent a variety of taxonomic groups, such as marine and terrestrial invertebrates, fish, and mammals.

The present detailed review paper fulfills one of the EPA's objectives in its validation process, namely, to summarize, explain, and document the relevant principles, methods, and techniques for a two-generation reproductive/developmental toxicity test using an invertebrate species of mysid shrimp for evaluating effects of potential endocrine-disrupting chemicals. After reviewing the current literature, the report recommends an initial Tier 2 protocol and an organism that will best meet the needs for testing; and it identifies issues that could require prevalidation studies.

The preferred mysid species is *Americamysis bahia*, because it is commercially cultured and readily available year-round, it has been the subject of many toxicity tests, it has a short generation time, and its testing requirements and biology are well known. One disadvantage to use of this mysid is that it is not indigenous to every geographic area that could be of interest. The exposure protocol recommended for the mysid testing is based on two existing protocols (EPA 1996, ASTM 1997) that we modified to allow two generation testing of *A. bahia*.

Because potential endocrine disruptors could elicit more than one response, and the responses may vary with the chemical tested, several endpoints are included in the testing program. Recommended endpoints for mysid endocrine disruptor experiments should be survival, growth rate, and specific reproductive output (time to first brood, viability of offspring, clutch size, and sex ratio). These endpoints in the recommended two-generation method should provide adequate information on the adverse consequences of a putative endocrine disrupting chemical to this representative invertebrate.

2.0   INTRODUCTION

2.1   DEVELOPING AND IMPLEMENTING THE ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP)

In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) mandated the United States Environmental Protection Agency (U.S. EPA) to screen substances found in drinking water sources of food to determine whether they possess estrogenic or other endocrine activity (Federal Register, 1998a, 1998b). Pursuant to this goal, the U.S. EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect..." (FQPA, 1996). The U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC, 1998), the U.S. EPA established the Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, i.e., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial substances, and environmental contaminants. To date, the U.S. EPA has implemented the program on two fronts: (1) the development of the Endocrine Disruptor Priority Setting Database, and the approach that will be used to establish priorities for screening compounds, and (2) prevalidation and validation studies of some of the Tier 1 and Tier 2 assays that are likely to be included in the testing battery. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) has been set up to advise and review new and ongoing work in the validation of these assays.

2.2   THE VALIDATION PROCESS

The U.S. EPA (and EDMVS) chose to follow the validation process established by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), of which the U.S. EPA was a charter member, for validation of the EDSP screening and testing methods. ICCVAM was established by the National Institute of Environmental Health Sciences (NIEHS) as a standing interagency committee to aid in the validation, acceptance, and harmonization of test methods designed to reduce animal use, refine procedures involving the use of animals so that they would experience less stress, and to replace animal tests whenever appropriate (ICCVAM, 2000). To this end, ICCVAM defined a flexible, adaptable framework for test method validation that was applicable to conventional and alternate methods, and could be applied to the needs of different agencies and regulatory processes.

The purpose of the validation is to establish the reliability and relevance of a test method with respect to a specific use. The process is science-driven, and addresses the scientific principles of objectivity and experimental design (NIEHS, 1997). In addition, as stated in the

ICCVAM report, "A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose." (NIEHS, 1997).

The validation process consists of four discrete phases: (1) initial protocol development, (2) prevalidation studies, (3) validation studies, and (4) external scientific peer review. The initial protocol, developed from existing information and experience (past and current research), serves as the starting point for initiating the validation process. Prevalidation studies consist of further development and optimization of specific initial protocols through targeted investigations. Either before or during prevalidation, a detailed review paper addressing all critical areas outlined in *Validation and Regulatory Acceptance of Toxicological Test Methods* (NIEHS, 1997) is prepared for each method to summarize, explain, and document decisions regarding the relevant principles, methods, and techniques recommended for the initial protocol. Targeted prevalidation investigations are designed to address questions necessary for completing an optimized, transferable protocol suitable for interlaboratory validation studies. Validation studies consist of comparative interlaboratory studies to establish the reliability and relevance of the protocols developed in the prevalidation stage. Validation requires the development of a detailed review paper to document what is known about the assay system proposed for validation.

A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose. The measurement of a test's reliability and relevance are independent stages in the validation of a test method, and both are required. Reliability is an objective measure of a method's intra- and interlaboratory reproducibility. If the test is not sufficiently reliable, it cannot be used for its intended purpose. Alternatively, if the test is not relevant, of questionable relevance to the biological effect of interest, or if it is not an appropriate measure of the effect, its reliability is academic. The relevance of a test may be linked to the mechanism of the toxic effect it measures and to its proposed uses (NIEHS, 1997). The studies conducted will be used to develop, standardize, and validate methods, prepare appropriate documents for peer review of the methods, and develop technical guidance and test guidelines in support of the EDSP.

Following the validation studies, results of an external scientific peer review of the study and the optimized protocols will be used to develop the U.S. EPA test guidelines.

2.3 PURPOSE OF THE REVIEW

The purpose of this detailed review paper (DRP) fulfills the objective of the validation process to define the purpose of the validation study for a two-generation reproductive/developmental toxicity test using an invertebrate species of mysid shrimp for evaluating effects of potential endocrine-disrupting chemicals. The DRP will summarize, explain, and document the relevant principles, methods, and techniques; it will recommend an initial Tier 2 protocol that will best meet the needs for testing; and it will identify issues that could require prevalidation studies.

Tier 2 is the final phase of the screening and testing program and therefore should provide more detailed information regarding the adverse consequences from endocrine disruption activity of a tested chemical or mixture. To fulfill this purpose, tests are often longer-term studies designed to encompass critical life states and processes, a broad range of doses, and administration by relevant route of exposure. In addition, the effects associated with EDCs can be latent and not manifested until later in life or may not be apparent until reproductive processes occur in an organism's life history. Thus, tests for endocrine disruption often encompass two generations to address effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity. The results from the Tier 2 testing should be conclusive in documenting a discernable cause-and-effect relationship of chemical exposure to measurable manifestation in the test organisms.

Standardized methods for mysid life cycle testing exist (EPA 1996, ASTM 1997). The modification of these methods that will be recommended in this report (Section 11.0) will be designed to:

- assess whether effects are a primary or secondary disturbance of endocrine function
- establish exposure/concentrations/timing and effects relationships
- be sensitive
- assess relevant endpoints
- include a dose range for full characterization of effects
- adhere to good laboratory practices
- be suitable for validation

Invertebrates (especially arthropods such as insects and crustaceans) constitute the vast majority of animal species on earth, and mysids represent an important and diverse group within the crustacean class. Although many invertebrate toxicity test protocols are routinely used in regulatory toxicity testing, few have been designed with endocrine-specific endpoints in mind. Although the growth, reproduction, development, and other aspects of invertebrate physiology and life cycle are known to be regulated by endocrine control, the endocrine systems and the hormones produced and used in the invertebrate body are not directly analogous to those of vertebrates. For example, ecdysone is a steroid hormone that regulates growth and molting in arthropods, and exhibits some functional and structural similarities to estrogen. Another aspect of morphogenetic and reproductive development of some arthropods is controlled in part by juvenile hormone (JH). Methyl farnesoate is a JH-like compound that could possibly play a role in reproduction and development (Borst et al. 1987; Laufer et al. 1987a, 1987b). Therefore, a method for testing mysids for effects of EDCs is relevant to assess the adverse consequences of chemicals demonstrated by weight of the evidence to be endocrine active in Tier 1 assays.

2.4   METHODS USED IN THIS ANALYSIS

In Appendix A, a detailed description of the methods employed for the literature search (e.g., key words, databases, and results) is provided. After key papers were identified, retrieved, and read for content, pertinent information was synthesized to create this DRP. At the back of

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this report is a CD ROM that has the Reference Manager Database of all documents reviewed. This database includes the reference citation and abstract.

2.5 ACRONYMS AND ABBREVIATIONS

Table 2-1 lists the acronyms and abbreviations used in the DRP, with the exception of commonly used units, such as h for hour, or L for liter. Each of the acronyms and abbreviations is also introduced at first use in the text.

Table 2-1. Acronyms and Abbreviations

20-E	20-hydroxyecdysone
4NP	4-nonylphenol
AFDW	ash-free dry weight
ASTM	American Society for Testing and Materials
BPDH	black pigment-dispersing hormone
CHH	crustacean hyperglycemic hormone
CYP	cytochrome P450 enzyme
DAH	dark adapting hormone
DDT	dichlorodiphenyl trichloroethane
DES	diethylstilbestrol
DRP	detailed review paper
EC	effects concentration
EC_{50}	median effective concentration
EDC	endocrine-disrupting chemical
EDMVS	Endocrine Disruptor Methods Validation Subcommittee
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EPA	United States Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act
GIH	gonad-inhibiting hormone
GSH	gonad-stimulating hormone
HPV-inerts	high production volume inert compounds
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IC	inhibition concentration
IGR	insect growth regulator
JH	juvenile hormone
LAH	light-adapting hormone
LC	lethal concentration

9

Table 2-1. Acronyms and Abbreviations (Contd)

LC_{50}	median lethal concentration
LOEC	lowest observed effects concentration
MAR	metabolic androgenization ratio
MIH	molt-inhibiting hormone
MSD	minimum significant difference
NIEHS	National Institute of Environmental Health Sciences
NOEC	no observed effect concentration
NPPE	nonylphenol polyethoxylate
OECD	Organization for Economic Cooperation and Development
OPPTS	EPA Office of Prevention, Pesticides and Toxic Substances
PCB	polychlorinated biphenyl
PoA	ponasterone A
SAB	Scientific Advisory Board
SAP	Scientific Advisory Panel
SDWA	Safe Drinking Water Act
TBT	tributyltin

3.0 OVERVIEW AND SCIENTIFIC BASIS OF MYSID LIFE CYCLE TOXICITY TEST

Many anthropogenic pollutants eventually end up in the world's oceans, carried there through riverine and estuarine pipelines (Nimmo and Hamaker 1982). Since the mid-1990s, there is an increased awareness that many sewage constituents or chemicals associated with industrial production that enter the environment can disrupt endocrine systems, and that these compounds will likely affect marine organisms (Depledge and Billinghurst 1999; Oberdörster and Cheek 2000). Although early concern over EDCs focused on vertebrates, attention recently has broadened to include invertebrates because of their ecological importance. Estuaries, which are intrinsically and commercially important ecosystems, are among the earliest recipients of EDCs. Among the many estuarine organisms that could be adversely affected by these compounds, crustaceans are good candidates for study of potential impacts. Crustaceans often are among the most abundant organisms in estuaries, and they form vital links in estuarine food webs. They are also susceptible to the effects of EDCs.

Many insecticides are considered to be EDCs, because they are specifically formulated to attack insect endocrine systems, affecting in particular the systems that are involved in molting and larval metamorphosis (Oberdörster and Cheek 2000). Most of these insecticides are JH analogues (Oberdörster and Cheek 2000). Although insects and crustaceans represent two classes within Phylum Arthropoda and consequently exhibit many similarities as well as differences, several recent studies showed that insecticides formulated as JH analogs adversely affect crustacean larvae by disrupting molting and metamorphosis (e.g., see McKenney and

Celestial 1996, which focuses on mysids). Crustaceans probably do not synthesize JH (LeBlanc et al. 1999); however, they do produce methyl farnesoate in the mandibular gland, and it is likely a natural JH analog. It is known to be involved in crustacean reproduction, but its specific role is uncertain (LeBlanc et al. 1999).

Crustaceans are important organisms to include in the evaluation of the adverse consequences of EDCs, and the selection of suitable species is the focus of the present review. Early studies of the effects of EDCs on estuarine crustaceans, most of which have occurred within the last few years, have focused on three primary groups, barnacles, copepods and decapods (Ingersoll et al. 1999, Hutchinson 2002). The results of these studies showed that some crustacean groups may be affected by exposure to EDCs, but others may not and, therefore, extrapolation of the results from testing one crustacean group to another is problematic. Billinghurst et al. (1998, 2000) examined the effects of two estrogens, 4-n-nonylphenol and 17β-estradiol on larval settlement and the production of a larval storage protein (cypris major protein, CMP) in *Balanus amphitrite*. Cyprids use CMP during settlement and the early post-settlement development. Because CMP is structurally related to vitellin, which is analogous to vitellogenin, it can be used as a biomarker of estrogen exposure in lower vertebrates. The expectation in these studies was that cyprid settlement might be affected by the stimulation of CMP synthesis after larval exposure to environmental estrogens. The results of the 1998 study, however, showed reduced settlement after exposure to both estrogens, but also showed that the cause was not related to endocrine disruption. The second study (Billinghurst et al. 2000) measured levels of CMP and found that they were elevated after exposure of nauplii to low levels of the estrogens. Hutchinson et al. (1999a, b) found that exposure to several steroids had no effect on the survival and development of copepod (*Tisbe battagliai*) nauplii and cautioned against extending the reported effects of steroid exposure in some species of crustaceans to the group as a whole. At about the same time, Bechmann (1999) showed that high levels (>62 μg/L) of nonylphenol were acutely toxic to *T. battagliai*, but that exposure to a low level (31 μg/L) did not affect any of the measured life-table parameters (survival, sex ratio, fecundity) measured. Brietholz and Bengtsson (2001) did not find evidence of endocrine disruption in the harpacticoid copepod *Nitocra spinipes* after exposure to the estrogens 17β-estradiol, 17α-ethynylestradiol, and diethylstilbestrol.

Decapods probably have been the subjects of more endocrine function and possible EDC effects-related testing than any other crustacean group (Fingerman et al. 1998, Ingersoll et al. 1999). Two species, the mud crab (*Rhithropanopeus harrisii*) and the grass shrimp (*Palaemonetes pugio*), have been the primary subjects for many of the endocrine studies and have been advocated as being potentially useful in studies of EDCs (McKenney 1999). Several studies have investigated the effects of EDCs on crustacean life cycles using decapod larvae as the test organisms (e.g., Lee and Oshima 1998; McKenney et al. 1998; Nates and McKenney 2000). Several studies (reviewed by McKenney 1999) have reported effects of the exposure of decapod larvae to JH analogs suggestive of the interruption of endocrine processes, but direct links were not established. More recently, Nates and McKenney (2000) found that exposure to the pesticide fenoxycarb disrupted lipid metabolism in mud crab larvae and suggested that the compound could be interfering with the endocrine regulation of lipid metabolism. Some

evidence of endocrine disruption in decapods was provided by Snyder and Mulder (2001) who found that exposure of lobster (*Homarus americanus*) larvae to the pesticide heptachlor altered ecdysteroid hormone levels that were linked to delays in molting.

Decapods are particularly appealing to EDC studies because much is known about their endocrine systems and their free-swimming larvae are likely to be susceptible to JH analogs and other Insect Growth Regulators (IGRs) produced to control insects (McKenney 1999). However, their relatively long generation time (usually >5 months, Ingersoll et al. 1999) makes decapods unlikely candidates for multigenerational testing.

There are few studies of the effects of EDCs on other estuarine crustacean groups. Brown et al. (1999) found that exposure to 4-nonylphenol reduced growth in the gammaridean amphipod *Corophium volutator*, but that this was probably a general response to the exposure rather than an interaction with molting hormones. They also reported that males exposed to 4-nonylphenol had larger second antennae than those in control treatments and suggested that the compound may have acted on the androgenic gland.

Mysid crustaceans have been used in regulatory (and other) toxicity testing for more than 20 years. Standard testing protocols have been developed for some species. Despite the fact that little is known about general endocrine functions in mysids (Ingersoll et al. 1999) and there have not been any direct links between potential EDCs, beyond certain IGRs, and endocrine disruption in mysids, they have been suggested as providing a useful model of the hormonal control of crustacean molting (Cuzin-Roudy and Saleuddin 1989). McKenney and Celestial (1996) studied the effects of methoprene, which is a JH analog used to control mosquitoes, on *Americamysis bahia* and found that mysids grown at sublethal concentrations were smaller, had a longer time to the production of the first brood, and produced fewer young per female than control animals. They suggested that the effects shown were likely from the interruption of endocrine function by the methoprene. Recently, tests of effects of EDCs on mysids (*Neomysis integer*) were reported by Verslycke et al. (2002).

Mysids, despite their superficial resemblance to shrimp, are more closely related to amphipods and isopods (Brusca and Brusca 1990). Mysids, amphipods, and isopods are grouped in the Superorder Peracarida, which is characterized in part by the retention of developing young in a marsupial brood pouch. All three taxa would be good candidates for toxicological testing, and amphipods and mysids are routinely used. However, for EDC testing, especially for life cycle tests, mysids offer clear advantages over amphipods. Most marine amphipods used in toxicological testing must be collected from their natural habitats just prior to use in tests. Although they can be held for a few weeks prior to testing, they generally are not cultured for tests. Currently, only one marine amphipod, *Leptocheirus plumulosus*, has been cultured successfully and used for growth and reproduction tests (EPA 2001). Conversely, several mysid species have been cultured in the laboratory and used in such life cycle tests.

Mysid crustaceans are distributed from 80°N to 80°S and occur in most aquatic environments, including brackish, freshwater, and marine. The feeding habitats of mysids

include consumption of detritus, phytoplankton, and zooplankton. Mysids are very important in freshwater, estuarine, and marine food webs. Stomach-content analysis revealed that mysids are a staple food for striped bass, tidewater silverside, and several species of flounder (Gentile et al. 1983).

For crustaceans, biological processes are regulated by a complex endocrine system (Cuzin-Roudy and Saleuddin 1998). Basically, inputs from the environment are integrated by a central nervous system. Neurotransmitters and neuromodulators govern the release of neuropeptides, which govern the production of hormones by the endocrine glands (Cuzin-Roudy and Saleuddin 1998). Molting, for example, is controlled by the release of molting hormones, which are ecdysteroids, and by neurosecretions for the central nervous system, which are accumulated and released by the sinus gland. The main endocrine centers for crustaceans described to date include the Y-organ, mandibular organ, androgenic gland, X-organ, and sinus gland.

The endocrine system of an invertebrate differs from that of a vertebrate organism both in the type of endocrine glands present and in the chemical structure (and consequently in the function) of specific hormones that are produced. Vertebrates produce estrogen, androgen, and thyroid hormones; whereas invertebrates do not. For example, crustaceans and most other invertebrates produce di- and tri-iodothyronine, but have no thyroid gland, and the function of the thyronines is unknown. Crustecdysone is in some ways analogous to a vertebrate's estrogen hormone, but it is structurally, functionally, and metabolically different from the vertebrate hormone (J.M. Neff, personal communication, January 15, 2002). Therefore, the response of an invertebrate animal to an EDC could be expressed differently than it would be in a vertebrate.

3.1   ECDYSTEROID SENSITIVITY TO MEASURED ENDPOINTS

Ecdysteroids are the molting hormones in crustaceans. These hormones also function in the control of reproduction and embryogenesis (Subramoniam 2000). Ecdysteroids are synthesized by the ecdysial glands or Y-organs. The Y-organ secretes ecdysone, which is converted to 20E, 3-dehyroxyecydsone, and 25-deoxyecdysone, which is a precursor to ponasterone A (PoA), the most dominant serum ecdysteroid in the premolt stage (Subramoniam 2000). Other sources for ecdysteroids are the ovary, epidermis, and the oenocytes (Delbecque et al. 1990). In many crustaceans, molting, and hence somatic growth, continue after maturity, with the result that the Y-organ is active in adults. For most crustaceans, growth and reproduction can be grouped into three functional categories. In the first category, represented by crab and lobster, reproduction occurs after a long intermolt period. The second category includes isopods and amphipods, the growth and reproduction of which are concurrent. The last category relates to the rapidly molting cirripedes, for which reproduction requires several molt cycles. Molting and limb regeneration are intertwined (Fingerman et al. 1998). When limb regeneration occurs, first a limb bud develops within a layer of cuticle, and then becomes free and unfolds when ecdysis occurs as part of the molting process. Synthesis and secretion of ecdysteroid by the Y-organs is inhibited by the molt-inhibiting hormone (MIH) from the sinus gland.

Among the mysids, there is synchronization between reproduction and molting. Accumulation of ovary ecdysteroid takes place during the premolt stage, when the hemolymph ecdysteroid levels rise sharply. It is presumed that the hemolymph ecdysteroids are transported to the ovary along with the yolk precursor material. This trend is seen in other species as well, and shows that the Y-organ is active during premolt and that it produces ecdysteroids that are transported to the ovaries. This observation was confirmed by Subramoniam (2000). A Y-organ ablation was performed on the shrimp, *Lysmata seticaudata*, which caused a subsequent depression of vitellogenin synthesis and retardation in ovarian growth. Further findings on the same shrimp revealed a failure of folliculogenesis, which is a necessary prerequisite for vitellogenin-uptake by oocytes during secondary vitellogenesis.

Methyl farnesoate (see Section 3.0) is also secreted by a mandibular organ; there is evidence that this compound is involved with the control of ecdysteroid synthesis. When a mandibular organ was experimentally implanted into the shrimp, *Penaeus setiferus*, there was a subsequent shortening of the molt cycle (Subramoniam 2000). Secretion by the Y-organ is controlled by methyl farnesoate, whereas inhibition is exercised by MIH from the X-organ sinus gland. The mandibular organ has also been implicated in the control of reproduction in crustaceans. Mandibular organ implants stimulated ovarian growth in the juvenile spider crab females and methyl farnesoate levels increased in the hemolymph and the mandibular organ during vitellogenesis in the crab, suggesting that this compound has a gonadotropic role similar to that of JH in insects. However, other studies showed no methyl farnesoate level effects within the vitellogenic period in the lobster, for example (Subramoniam 2000).

In general, there is very little published research that documents the effects of ecdysteroids on mysids. One current project funded by the EPA and conducted by the University of South Carolina is in the process of evaluating environmentally mediated endocrine disruption in three estuarine crustaceans (Chandler et al. 2000). One species of shrimp, *Palaemonetes pugio*, was exposed to a concentration-series of endosulfan, an endocrine active chemical responsible for many fish kills. The result was reproductive impairment of the shrimp with increasing endosulfan concentration. Clutch size and time to hatch were not affected.

Recent advances in biological control agents to control insect pests have inspired the synthesis of insect growth regulators (IGRs), which find their way into the estuarine environment by either direct or indirect application. Crustaceans, which along with insects are in the phylum Arthropoda, could also be sensitive to these compounds. The mysid group has been shown to be among the most sensitive members of the estuarine community (McKenney 1982, 1985, 1986, 1996; Nimmo and Hamaker 1982; Nimmo et al. 1981). One study focused on exposure of *Americamysis bahia* to methoprene, a JH analogue (McKenney and Celestial 1996). The goal was to determine whether typical application rates shown to control mosquito larvae also cause problems for nontarget organisms. The results showed a significant effect during the mysid life cycle test. Total lethality occurred at 125 μg in a 14-day test. Similar concentrations caused significant mortality in the larvae of an estuarine crab and shrimp. Other sublethal endpoints, such as reduced growth (weight), longer time to first brood, and a significant reduction in brood size were also observed. These results suggest that methoprene could interfere with the

endogenous endocrine system, which uses hormones that act like JH. Retarded growth rates were also accompanied by bioenergetic disruption, resulting in lower net growth efficiency values. This suggested that increased metabolic demands reduced the amount of assimilated energy available for new tissue production (McKenney 1982, 1985). The delays in mysid first brood production could be the result of slowing sexual maturity and/or embryogenesis. Diminished reproductive success could be the result of inhibited vitellogenesis, modifications in ovarian development, or disruption of successful embryogenesis. In either case, further work is required with the mysid to determine a more conclusive cause-and-effect relationship between potential EDCs and their effects, as observed by test measurement endpoints.

Metals such as mercury, cadmium, and zinc have been reported to affect molting and limb regeneration in crabs. Organic compounds such as Aroclor 1242 and sodium pentachlorophenate reportedly had a similar effect—inhibition of limb regeneration—in the grass shrimp, *Palaemonetes pugio*, but had no effect on the molting cycle. This suggests that these chemicals act directly on limb development, but not on the hormonally controlled molting cycle (Fingerman et al. 1998).

Baldwin et al. (2001) documented the effects of several EDCs on the freshwater crustacean, *Daphnia magna*. One experiment focused on exposure to 20-hydroxyecdysone (20-E), the crustacean molting hormone, and to PoA, an endogenous hormone that has 20 times higher affinity for the ecdysone receptor. The 21-day exposure had little effect on reproduction for either compound, except at the highest concentrations. However, adults suffered high mortality rates and either did not produce broods or produced smaller broods. Second-generation effects were not observed as a result of 20-E exposure, but there was a slightly significant effect on reproduction from PoA exposure. The effect on reproduction could be attributed to the structure of PoA: it has fewer hydroxyl groups, and could be less easily metabolized as is 20-E. The resulting longer exposure could allow second-generation effects. It is also possible that the higher affinity for PoA to ecdysone receptor sites caused a limited effect on secondary vitellogenesis in developing daphnids, which manifested itself as reduced reproduction due to smaller brood size (Baldwin et al. 2001).

4.0 CANDIDATE MYSID TEST SPECIES

Several characteristics should be considered in determining the most appropriate species for use in EDC-related testing. Nimmo and Hamaker (1982) and Roast et al. (1998) suggested that the test organism should be ecologically relevant, and sensitive to contaminants. Because it must be available as needed for testing, it must be either abundant and easily collected, or amenable to laboratory culture. Its diet should be well understood and easy to provide in the laboratory, therefore allowing it to be well-adapted to laboratory conditions and reducing the need for extensive acclimation periods. The ideal test organism should have a short, relatively simple life cycle that allows for the testing of successive generations. Nimmo and Hamaker (1982) and Roast et al. (1998) argued that mysids fulfill these requirements well.

Ingersoll et al. (1999) included several additional characteristics that are important considerations in the selection of test species. Some of the features, for example mode of reproduction and knowledge of endocrinology, do not allow for discrimination among candidate mysid species. Important to selecting a candidate mysid species for use in the multigenerational EDC testing program are the ability to culture the species in the laboratory (with a strong probability that transgenerational testing is possible), generation time should be relatively short, allowing for full life-cycle testing, size (larger animals provide more tissue for measuring hormone titers, but usually have longer generation times), and the availability of standard (consensus-based) testing methods, including whether or not new methods must be developed to measure EDC-relevant endpoints.

Several mysid species are considered below for their potential utility in EDC testing. For each, a discussion of natural history, availability, and culture and handling is offered, along with a summary paragraph on strengths and weaknesses of the species as test organism.

4.1 *AMERICAMYSIS BAHIA*

Americamysis bahia is a small mysid crustacean that occurs in coastal estuaries and embayments ranging from the Gulf of Mexico to Narragansett, Rhode Island (Price et al. 1994).

4.1.1 Natural History

A. bahia typically occurs in areas where the salinity is >15‰, but is more abundant in higher-salinity habitats. Molenock (1969) originally described the species as *Mysidopsis bahia*, but Price et al. (1994) transferred it to a new genus, *Americamysis*, during their taxonomic revision. The natural history of the species is well known; the following description is based on Weber (1993). Adults of *A. bahia* may reach almost 10 mm in total length, and females attain a larger size than males. Individuals become sexually mature in about 12 to 20 days, and the genders can be distinguished when the animals reach about 4 mm in total length, at which time the brood pouch typically has started development. At about 12 days, the female's ovaries begin to contain eggs, and the brood pouch is fully formed at about 15 days. The developing young are carried in the brood pouch for an additional 2 to 5 days, resulting in a life cycle of about 17 to 20 days. New broods may be produced about 4 to 7 days. Females produce an average of about 11 eggs per brood, and the number is directly related to female body length.

4.1.2 Availability, Culture, and Handling

A. bahia is cultured commercially by many laboratories located throughout the United States and therefore is readily available to testing laboratories. With only a few days' notice, commercial suppliers can ship <24-h-old mysids via overnight service, allowing testing laboratories time to acclimate the animals to test conditions. However, testing laboratories can also easily culture the species. The mysids can be raised in 80-L to 200-L aquaria provided with continuous flow-through or recirculating systems. The water temperature and salinity within the culture tanks are typically 24°C to 26°C and 20‰ to 30‰, respectively. The cultures are fed

newly hatched brine shrimp (*Artemia* sp.). Several sources provide information on culturing this species (e.g., Lussier et al. 1988). Although a small species, individuals of *A. bahia* are relatively easily handled.

4.1.3 Strengths and Weaknesses

As a candidate test species, *A. bahia* has many strengths and few weaknesses. Its primary advantages include its widespread availability and ease of culture. Because animals can be obtained for commercial cultures, the likelihood of misidentifying the species is very low. Its relatively short generation time makes it desirable in life cycle testing. The species is widely used in toxicological testing, and appropriate test conditions are well known. Standardized life cycle test protocols have been developed (EPA 1996; ASTM 1997) and applied (e.g., McKenney 1986, 1994; McKenney and Celestial 1996) or evaluated (Lussier et al. 1999). Included among the standardized test protocols are many of those necessary to measure EDC-related endpoints; others appear in peer-reviewed publications (Ingersoll et al. 1999). More recently, studies showed that the results from toxicity tests, which included standard and multigenerational tests using *A. bahia*, could be used to extrapolate from laboratory to population effects (Kuhn et al. 2000; 2001). One criticism of the widespread use of *A. bahia* in toxicological testing is that because it is a warm-temperate or subtropical species, it may not be ecologically relevant to colder-water materials testing.

4.2 *HOLMESIMYSIS COSTATA*

Holmesimysis costata, previously referred to as *Acanthomysis sculpta*, is an ecologically important species that ranges from southern California to British Columbia (Hunt et al. 1997).

4.2.1 Natural History

H. costata is a dominant member of the plankton community living within the surface canopy of the giant kelp, *Macrocystis pyrifera* (Chapman et al. 1995). Adults may reach lengths of about 7 mm to 13 mm (Daly and Holmquist 1986), and females attain a larger size than males (Turpen et al. 1994). Sexual maturity occurs at about 42 days, at which time males, which are recognized by an extended fourth pleopod, can be distinguished from females, recognized by the developing brood pouch (Turpen et al. 1994). Young are released when the females are about 65 to 73 days old. Brood size among laboratory-cultured females averaged about 16 released juveniles per female; in contrast, field-collected females released substantially larger broods of 27, on average (Turpen et al. 1994). Brood size was directly related to female size. Turpen et al. (1994) also reported that all laboratory-reared females died before releasing a second brood of young.

4.2.2 Availability, Culture, and Handling

Field-collected animals are available from a few suppliers and are likely to be available year round, but the species is not cultured commercially (Turpen et al. 1994). Field-collected

mysids can be cultured in the laboratory, and guidelines for doing so have been established (Chapman et al. 1995). However, broodstocks should be rejuvenated periodically by the addition of field-collected animals (Turpen et al. 1994). Culture tanks can range in volume from 4 L to 1000 L and should be provided with aeration and fronds from the giant kelp, *M. pyrifera* (Chapman et al. 1995). Adults are fed newly hatched *Artemia*, whereas juveniles are fed *Artemia* supplemented with a small amount of ground fish-food flakes (e.g., Tetramin). The animals are easily handled by using a combination of small-mesh dip nets and pipettes to transfer them from culture tanks to test chambers.

4.2.3 Strengths and Weaknesses

The primary strengths of this species are its ecological relevance to northeast Pacific regional testing conditions, its relatively large brood sizes, and its ease of handling and maintenance. The species has been used in several toxicological tests (e.g., Singer et al. 1998) and standardized test protocols for the species have been developed (Chapman et al. 1995) and evaluated (Hunt et al. 1997). The principal disadvantages inherent in using *H. costata* are its long generation time (~70 days) and the difficulty in raising multiple broods in the laboratory. The tests required to measure many EDC-related endpoints must be developed (Ingersoll et al. 1999) or are impractical because of the species long generation time and the difficulty in raising multiple broods. Because the original animals are field-collected, they must be identified carefully prior to their use in testing. Also, potential population-related differences in pollutant sensitivities should be assessed before comparing the results of tests performed with mysids from different populations.

4.3 *MYSIDOPSIS INTII*

Mysidopsis intii is an epibenthic species that occurs in the eastern Pacific from South America to the southern California coast of the United States (Price et al. 1994; Langdon et al. 1996).

4.3.1 Natural History

M. intii has only recently been reported from the United States (off Los Angeles), but it could be more widespread (Langdon et al. 1996). *M. intii* is a relatively small species that attains a body length of about 6 mm to 7 mm. The genders can be distinguished at about 9 to10 days after hatching. Eggs enter the brood pouch at about 13 days, and juveniles are released at about Day 20 (Langdon et al. 1996).

4.3.2 Availability, Culture, and Handling

Animals to be used in testing must be obtained from field collections, because there is no commercial culture of the species. Individuals of *M. intii* are collected by using an epibenthic sled, and the wild-caught animals are then used to establish breeding stocks in the laboratory. The species is easily cultured in the laboratory in 40-L to 90-L tanks continuously supplied with

flowing, filtered seawater. Langdon et al. (1996) determined that the optimal temperature for high juvenile production is 20°C. To ensure high reproductive output, adults should be fed recently hatched *Artemia* and adult copepods, *Tigriopus californicus* (Kreeger et al. 1991; Langdon et al. 1996). The *Artemia* diet can be enriched with fatty acid supplements, and the mysid cultures provided with fatty acid boosters (UCSC 1998). Separation of juvenile mysids from the adult cultures is easily accomplished by using light to attract them through a 1-mm-mesh divider into an isolation chamber (Langdon et al. 1996).

4.3.3 Strengths and Weaknesses

M. intii represents an indigenous, ecologically relevant species for testing contaminants that could negatively affect northeast Pacific coast ecosystems. The life cycle of *M. intii* is much shorter (~20 days) than that of *H. costata* (~70 days), the other Pacific coast species commonly used in toxicity testing. The EPA sponsored the development of a 7-day toxicity test protocol (Langdon et al. 1996) that has been applied (UCSC 1998) and evaluated (Harmon and Langdon 1996). The primary disadvantages associated with using *M. intii* are the lack of available commercial culture and the fact that testing protocols to measure EDC-related endpoints need to be developed. Regardless of the source of test animals, *M. intii* individuals used in testing must be identified carefully prior to their use in tests. Further, the requirement to supplement an *Artemia* diet with copepods (*T. californicus*), which initially must be field collected, could be an impediment to the use of *M. intii* by some laboratories. Finally, potential population-related differences in pollutant sensitivities should be assessed before comparing the results of tests performed on different populations.

4.4 *NEOMYSIS INTEGER*

Neomysis integer is found throughout northern Europe (Mees et al. 1994) and has been suggested as an appropriate species for use in European toxicity testing programs (Roast et al. 1998, 2000b).

4.4.1 Natural History

N. integer is a relatively large, hyperbenthic species that occurs in relatively low-salinity portions of estuaries (Roast et al. 2001). Females may attain a standard length of about 18 mm (measured from the base of the eyestalk to the end of the last abdominal segment); males are smaller (Mees et al. 1994). Brood size is strongly correlated with the size of the female: the number of larvae per brood extends to about 80 individuals for females of 16 mm or more in length (Mees et al. 1994).

4.4.2 Availability, Culture, and Handling

Because it is the dominant mysid inhabiting northern European estuaries (Mees et al. 1994), it is readily available, but animals to be used in testing must be field collected. It is not cultured commercially, but wild-collected animals are easily maintained in the laboratory.

4.4.3 Strengths and Weaknesses

The principal strength of this species for use in toxicity testing is that it is a common and ecologically important component of European estuaries. However, it is not commercially cultured, and all animals to be used in tests must be collected from estuaries and raised in the testing laboratories. Testing protocols to measure EDC-related endpoints need to be developed and studies addressing the utility of this species in EDC testing have recently been initiated (Verslycke and Janssen 2002, Verslycke et al. 2002). These animals must be identified carefully prior to testing. Also, potential population-related differences in pollutant sensitivities should be assessed before comparing the results of tests performed on different populations.

4.5 OTHER MYSID SPECIES

A few other species of *Americamysis*, including *A. almyra* and *A. bigelowi*, have been used in toxicity testing or related studies. *A. almyra* is closely related to *A. bahia*, and the two species have similar geographic distributions; however, *A. almyra* inhabits less saline waters (Price et al. 1994). The reproductive biology, including brood size and generation time, of the species is very similar to that of *A. bahia* (Reitsema and Neff 1980). The species is amenable to laboratory culture. Both recirculating (Reitsema and Neff 1980) and static (Domingues et al. 1998, 1999) culture systems have been developed. *A. bigelowi* is also biologically similar to *A. bahia* and to *A. almyra*. It occurs along the east coast of the United States from Massachusetts to Florida (Price et al. 1994). Although Gentile et al. (1982) found it to be suitable for use in toxicology testing, *A. bigelowi* has not received widespread attention as a test species.

Neomysis mercedis was advocated as an acute toxicity test organism appropriate for estuarine waters having low to intermediate salinities (Brandt et al. 1993). It occurs in freshwater and brackish waters from California to southern Alaska (Daly and Holmquist 1986) and recently has been used in toxicity testing (Farrell et al. 1998a, b; Hunt et al. 1999). A standardized acute toxicity testing protocol has been developed for *N. mercedis* (ASTM 1998). *N. mercedis* is not cultured commercially, but can be reared in the laboratory (Brandt et al. 1993). *N. mercedis* has a relatively long generation time of about 3 to 4 months (Brandt et al. 1993). The tests required to measure many EDC-related endpoints must be developed (Ingersoll et al. 1999) or are impractical because of the species long generation time.

5.0 EXPOSURE PROTOCOLS FOR REPRODUCTIVE AND DEVELOPMENTAL TOXICITY TESTS

Mysids are typically pelagic, and consequently have contact with EDCs through the water-column. The main route of exposure is through their swimming and feeding in the water-column (Roast et al. 1998). Many estuarine mysids are also hyperbenthic (Roast et al., 1998) and make diurnal migrations into the water column (Dauvin et al. 1994). For these reasons, mysids could serve as sensitive indicator species to monitor the effects of EDCs through exposure to either the water column or sediment or through dietary uptake.

5.1 ROUTE OF EXPOSURE

In a testing program, the routes of administration could be through spiking a potential EDC of interest into the water column or into clean sediment, or by mixing the compound with the mysid food source prior to feeding. Because of the particular diet of the mysids proposed for use, the two most practical routes are through the water column or sediment, as described below.

5.1.1 Water

To conduct toxicity tests designed to measure the effects of potential EDCs, chemicals of known purity are typically purchased from suppliers. The potential EDC of interest is mixed with the dilution water using a solvent carrier, if necessary (Lussier et al. 1985; Nimmo and Hamaker 1982). The dilution water should be acceptable to mysids, be of uniform quality, and should not unnecessarily affect results of the test. For detailed discussion of dilution water, see EPA (1996). If a solvent carrier other than dilution water is used, its concentration in the test solution should be kept to a minimum, and should be low enough that it does not affect the survival, growth, or reproduction of the mysids (EPA 1996). It is important that the highest concentration of the EDC not exceed the single-phase seawater solubility of the compound, when a solvent carrier is used. It could require direct measurement of the test chemical's solubility in clean, particle- and dissolved-organic-carbon-free seawater of the salinity to be used in the test.[1] Delivery systems are designed to provide either continuous or intermittent flow of the chemical and dilution water mixture (McKenney et al. 1991). The EDC is mixed with dilution water in the mixing chamber, agitated, and then delivered to the replicated test chambers.

Continuous-flow systems (Figure 5-1) are designed to deliver a constant concentration to the test chamber, often by means of the metered pump of a siphon-flush system, which produces a 50% exchange of volume every 4 h, with an incoming flow rate of 30 mL/min and an outgoing flow rate of 100 mL/min (Gentile et al. 1982). The chosen system should be calibrated prior to use to ensure that the appropriate concentration of the test substance is achieved in the test chambers. The general operation of the delivery system should be checked twice daily, with a target 24-h flow rate through the test chamber equal to at least five times the volume of the testing chamber. Also, the flow rates should not vary more than 10% between replicate chambers or over time (EPA 1996). Table 5-1 presents some examples of the types of systems used in toxicity testing, along with their advantages and disadvantages.

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[1]Dissolved organic carbon and particulate organic carbon in exposure water can substantially affect the amount of the toxicant in true solution and therefore most bioavailable to the test organisms. Dissolved organic carbon has a strong affinity for binding nonpolar organic compounds and some metals, which decreases their bioavailability. It also thereby could decrease the exposure concentration of the toxicants, and accordingly, influence the results of the bioassay. Therefore, there probably should be a requirement developed as part of the testing protocol for the acceptable concentration of dissolved and particulate organic carbon in exposure water, particularly when natural seawater is used (J.M. Neff, personal communication, January 15, 2002; ASTM 1997).
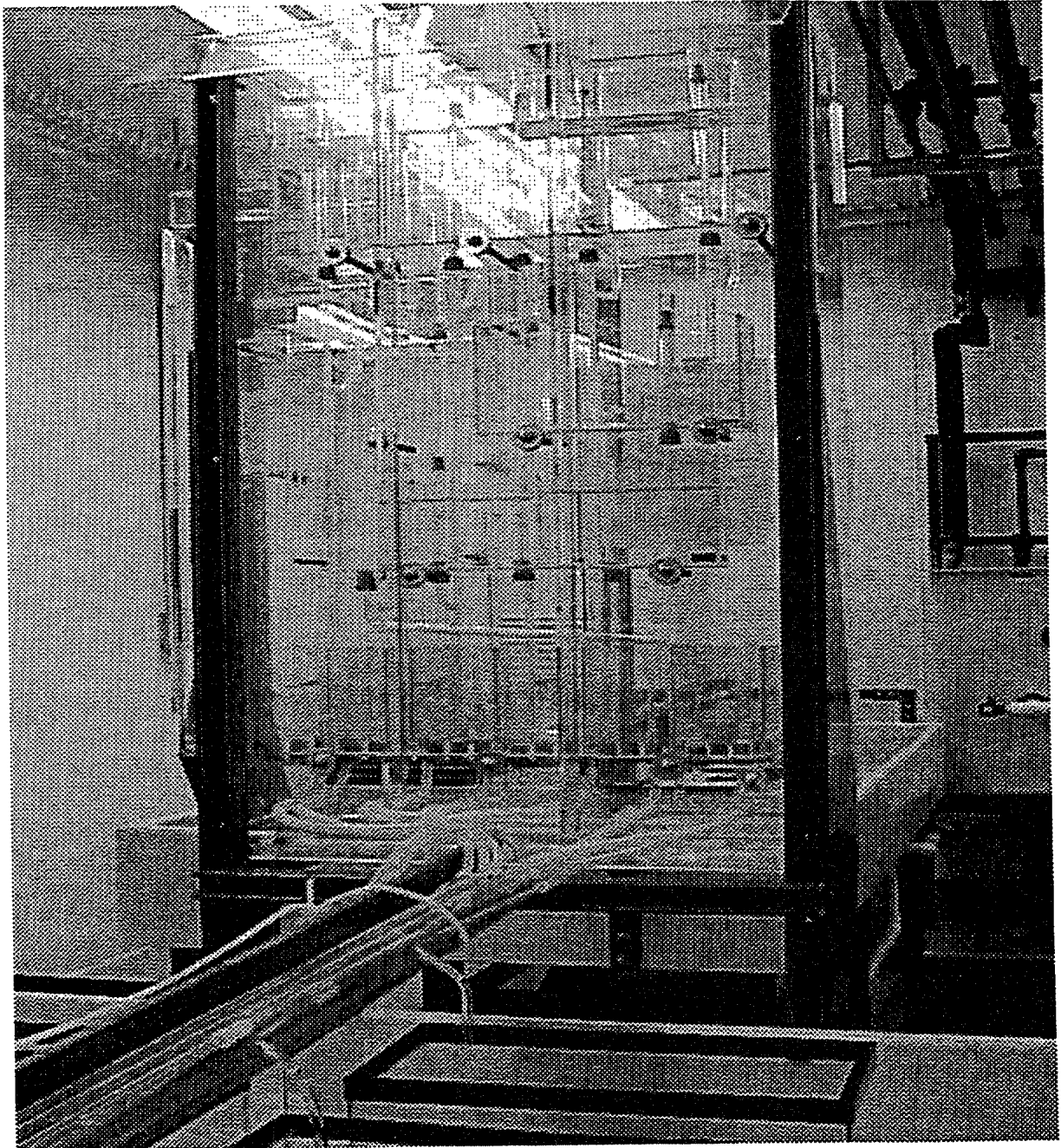
Figure 5-1. Diluter

Table 5-1. Types of Water Delivery Systems

Type	Description	Advantages	Disadvantages
Static	solution not changes during testing	no maintenance, inexpensive, performed in large numbers	toxicant concentrations may shift due to uptake by organisms, volatilization, decreased dissolved oxygen, or changes in pH.
Static Renewal	test solution changed at regular intervals using either a manual pump and freshly prepared replacement water	improved consistency of toxicant concentration	replacement of solution may stress the test organisms
Flow-through	solution replaced by automated system using a controlled dosing system a proportional diluter or a continuous flow siphon system	improved consistency of toxicant concentration, savings in labor hours	equipment can be expensive large volumes of test solution may require costly disposal

5.1.2 Sediment

Mysids are currently used in routine toxicity tests to examine the potential toxicity of marine sediment (Carr et al. 1998; Cripe et al. 2000). It has been documented (Cripe et al. 2000) that the mysids were observed to collect sediment, manipulate it at the mouth region, and drop it. This suggests that mysids could be used to test sediment suspected of containing EDCs.

5.2 CONCENTRATION SERIES

The goal of the chronic life cycle test should be to calculate endpoints such as survival, growth, or reproduction that are inhibited at a specific chemical concentration. If nothing is known about the sensitivity of the mysid to the chemical being tested, then a geometrically spaced series of test concentrations is used to establish a dose-response relationship for the key endpoints. If there is some information concerning the concentration-effect curve, then several other approaches could be taken:

- Conduct an acute range-finding test in which the organisms are exposed to a control and three or more concentrations of the test material that differ by a factor of 10. The test design for the acute test should use the same testing conditions and same age of organisms as the chronic test. The results of a water-only acute test could be used to calculate a more precise concentration series to target for the chronic test.

- Alternatively, estimation of concentration series could use an acute-to-chronic test ratio for a species of comparable sensitivity: the result of the acute test can be divided by the acute-to-chronic ratio. Generally, acute-to-chronic ratios determined with

saltwater mysids are often less than 5.

- If no other useful information is available, the highest concentration of test material in a life cycle test with mysids is often selected to be equal to the LOEC in a comparable acute test (ASTM 1997). Tests can also be used to generate the median lethal concentration (LC_{50}), median effective concentration (EC_{50}), or median inhibition concentration (IC_{50}), using a toxicant concentration series (0.5 or higher) that will provide partial mortalities at two or more concentrations of the test chemical (EPA 2000).

5.2.1 Statistical Considerations

Statistical methods are used to make inferences about populations based on samples from those populations. The goal of any statistical analysis is to provide a measure for assessing changes, relations, and anomalies in data, usually by comparing the response observed for a treatment relative to a control. During toxicity testing, each test chamber to which a single application of treatment is applied is a discrete experimental unit. Experimental units should be independent and should not differ systematically (ASTM 1997, Appendix).

Replication is the assignment of a treatment to more than one experimental unit. The variation among replicates is a measure of the within-treatment variation and provides an es-timate of within-treatment error for assessing the significance of differences between treatments. Four replicates are recommended for the chronic toxicity testing for each control or experimental treatment. All aspects of the test should be randomized to ensure that there is an unbiased assignment of treatments within a test system and to the exposure chambers, ensuring that no treatment is favored and that observations are independent (EPA 2000). The most common techniques for statistical analysis of data sets include graphical displays, pairwise comparisons, analysis of variance (ANOVA), multiple regression techniques, concentration-effect curve analysis, and multiple regressions (ASTM 1997, Appendix). Graphical displays should be produced every time a test is performed because the results could provide useful information relative to the structure of the data and reveal unanticipated relationships or anomalous data points (ASTM 1997, Appendix). Current computer software programs have made graphical estimations relatively easy.

When the assumptions of normality or homogeneity of variance are not met, transformations of the data may remedy the problem, so that the data can be analyzed by parametric procedures, rather than by a nonparametric technique. The first step in such an analysis is to transform the responses. Some endpoints, such as the proportion surviving, can be transformed by the arcsine-square-root, whereas others, such as growth and reproduction, can be transformed by logarithmic methods. When the test design requires the use of a solvent of dilution water control, then the data can be compared using either a t-test or other similar hypothesis test (EPA 2000).

Some experiments are set up to compare more than one treatment with a control, whereas others compare the treatments with one another. The basic design of both types experiments is similar. After the applicable comparisons are determined, the data must be tested for normality and whether the variances of the treatments are equal to determine whether parametric statistics are appropriate. If normality of the data and equal variances are established, then ANOVA can be performed to address the hypothesis that all the treatments including the control are equal (EPA 2000).

If the data are not normally distributed or the variances among treatments are not homogeneous, even after data transformation, nonparametric analyses are appropriate. If there are four or more replicates per treatment and the number of replicates per treatment is equal, the data can be analyzed with Steel's Rank Sum Test (Steel 1959). Unequal replication among treatments requires data analysis with the Wilcoxon Rank Sum Test with Bonferroni's adjustment. Steel's Rank Sum Test is a nonparametric test for comparing treatments with a control. This test is an alternative to the Dunnett's test and can be applied to data when the normality assumption has not been met. Steel's test requires equal variances across treatments and the control, but is thought to be fairly insensitive to deviations from this condition (EPA 1991). Wilcoxon's Rank Sum test is a nonparametric test to be used as an alternative to the Steel's test when the number of replicates is not the same within each treatment. A Bonferroni's adjustment of the pairwise error rate for comparison of each treatment versus the control is used to set an upper bound of α on the overall error rate. This is in contrast to the Steel's test with a fixed overall error rate for α. Thus, Steel's test is more powerful (EPA 1991).

The recommended procedure for pairwise comparisons that have a comparison-wise α error rate and equal replication is to perform an ANOVA followed by a one-sided Fisher's Least Significant Difference (LSD) test (Steel and Torrie 1980). A Duncan's mean comparison test should give results similar to the LSD. If the treatments do not contain equal numbers of replicates, the appropriate analysis is the t test with Bonferroni's adjustment. For comparisons that maintain an experiment-wise α error rate, Dunnett's test is recommended for comparisons with the control.

Commonly used approaches are the graphical, probit, trimmed Spearman-Karber, and the linear interpolation methods. Methods for evaluating point estimate data using logistic regression are outlined in Snedecor and Cochran (1989). In general, results from these methods should yield similar estimates. Each method is outlined below, and recommendations are presented for the use of each method.

Data for at least five test concentrations and the control should be available to calculate an LC_{50} although each method can be used with fewer concentrations. Survival in the lowest concentration must be at least 50%, and an LC_{50} or EC_{50} should not be calculated unless at least 50% of the organisms respond in at least one of the serial dilutions. When less than 50% response occurs in the highest test concentration, the LC_{50} or EC_{50} is expressed as greater than the highest test concentration.

The trimmed Spearman-Karber method is a modification of the Spearman-Karber, nonparametric statistical procedure for estimating the LC_{50} or EC_{50}, and the associated 95% confidence interval (Hamilton et al. 1977). This procedure estimates the trimmed mean of the distribution of the log_{10} of the tolerance. If the log tolerance distribution is symmetrical, this estimate of the trimmed mean is equivalent to an estimate of the median of the log tolerance distribution.

The linear interpolation method calculates a toxicant concentration that causes a given percentage reduction (e.g., 25%, 50%) in the endpoint of interest and is reported as an inhibition concentration (ICp value, where p = the percent effect). The procedure was designed for general applicability in the analysis of data from chronic toxicity tests and for the generation of an endpoint from a continuous model that allows a traditional quantitative assessment of the precision of the endpoint, such as confidence limits for the endpoint of a single test or a mean and coefficient of variation for the endpoints of multiple tests.

6.0 DESCRIPTION OF ASSAY ENDPOINTS REFLECTIVE OF REPRODUCTIVE AND DEVELOPMENTAL IMPAIRMENT

In considering the list of potential endpoints presented in the following sections, it is important to remember that one of the difficulties in EDC testing is ensuring that the measured response is directly related to exposure to the potential EDC and that the response is the result of interruption of endocrine function (Ingersoll et al. 1999). Although many, if not all of the endpoints described in the following sections could indicate a response to an EDC, most also will vary in response to exposure to other stressors. Further confounding the interpretation of testing results is the interrelatedness of some endpoint measurements. Thus, a stressor could act on a mysid to reduce its swimming ability, thereby reducing its ability to feed, which then reduces its growth. Similarly, reduced fecundity could be an expression of reduced growth. Often, exposure to a compound that results in reduced fecundity also results in reduced growth (Nimmo et al. 1981); however, this is not always the case. Lussier et al. (1999) reported fecundity effects without reduced growth, and McKenney and Celestial (1996) found reduced reproductive measures at concentrations of methoprene below that which resulted in reduced growth. Various endpoint measures can have differing sensitivities to stressors. For example, reproductive parameters are often, but not always, more sensitive measures of contaminant toxicity than is simple survival (Lussier et al. 1985). Hollister et al. (1980) found no reproductive impairment among test organisms, in spite of significant mortality due to exposure to alkaline effluent. Additionally, some reproductive measures can be affected by stressors, whereas others are not (e.g., Hollister et al. 1980; Lussier et al 1985). Clearly, the possible interrelationships among potential endpoints and the varying sensitivities shown by some endpoints underscore the need to use multiple endpoints in EDC testing.

6.1 GROWTH, MORPHOLOGICAL, AND BEHAVIORAL ALTERATIONS

Measures of growth, morphological changes, and changes in test organism behavior as indicators of EDC effects are reviewed below.

6.1.1 Growth

Molting is one of the key arthropod physiological processes that is under hormonal control, and therefore, it is susceptible to the negative effects of EDCs. Molting is regulated primarily by the interaction of molt stimulating hormones (ecdysteroids) and nervous system secretions produced in the cephalothorax with molt inhibiting hormones produced in the eyestalks (e.g., Cuzin-Roudy and Saleuddin 1989; Fingerman1997; Subramoniam 2000). Molting is either directly or indirectly involved in the expression of the various endpoints that may be examined through toxicological testing. Because noticeable growth can only occur as a result of molting, any disruption of molting could result in alterations in growth. Many pesticides, generally classed as Insect Growth Regulators (IGRs), have been developed that target insect development. IGRs function as ecdysone agonists, JH or anti-JH analogs, and chitin synthesis inhibitors. All can have detrimental effects on crustaceans, especially through interruption of the molt cycle, most likely by impacting endocrine function (Touart 1982). For this reason, the estimation of molting frequency may be a useful endpoint relevant to mysid EDC testing. The duration of the molt cycle of adult mysids (*Americamysis bahia*) was determined to be about 6.6 d (Touart 1982), with the duration for males being slightly less and that for females being slightly more. Juveniles are expected to molt within the first 24 h post release, but may delay molting until about 48 h (Touart 1982). Successive juvenile molts occur at increasingly longer durations. Touart (1982) found that at test conditions of 22 °C and 20 ‰, sexual characters were noticeable after the fourth molt (9 to12d) and that mating occurred after the fifth or sixth molts (17 to19d). Higher test temperatures would likely shorten the duration between molts. Touart also found that the pesticide diflubenzuron (Dimilin®) increased the duration of the molt cycle in *A. bahia*, probably acting on the mysid endocrine system as a molt inhibitor. This was demonstrated by his experiment in which eyestalk-ablated mysids that were then exposed to dimilin had molt cycle durations that did not differ from those in control animals. In effect, the Dimilin likely replaced or mimicked the "natural" mysid molt inhibitor synthesized in the eyestalks.

Two direct measures of growth may be used in the assessment of sublethal effects on mysids. Probably the most common measurement is the determination of simple dry weight. At test termination, animals are briefly rinsed in deionized water to remove salt, and dried to constant weight. In published studies that employ this method, drying temperatures ranged from 60°C (e.g., McKenney 1982) to 105°C (Khan et al. 1992) and drying times ranged from 16 h (Khan et al. 1992) to 72 h (UCSC 1998). ASTM (1997) specified that growth should be measured by drying animals at 60°C for 72 h to 96 h, or to constant weight. ASTM also recommended that separate determinations be made for males and females. Ash-free dry weight (AFDW) is often used as the appropriate weight measurement for many invertebrates, because the technique reduces any inaccuracies introduced by inorganic constituents in the organism's body. Inorganic components can originate from processes such as the development of skeletal components or feeding (the ingestion of sediment). As with some other small crustaceans, the small size of the mysids and their planktonic diet may make the removal of ash from the dry weight measurement an unnecessary step that would not greatly improve the accuracy of the measurement (EPA 2000).

Another direct measurement of growth is body length. Body length has been measured as the distance from the base of the eyestalks to the tip of the telson (Hunt et al., 1997; UCSC, 1998) or to the tip of the exopod (Langdon et al., 1996). ASTM (1997) suggests that body length should be measured along the midline of the body from the tip of the carapace to the tip of the exopod (excluding the terminal setae). ASTM notes that it is difficult to make this measurement on preserved animals because of the body curvature that results from the fixation process. Langdon et al. (1996) reduced the potential inaccuracy in this measurement by relaxing the mysids prior to fixation and then determining length as the sum of a series of relatively straight-line measurements.

The usual response to exposure to toxicants is reduced growth (several studies) although it may not be a particularly sensitive endpoint (Lussier et al., 1999). McKenney and Celestial (1996) reported reduced growth after exposure to methoprene, a juvenile hormone analogue and likely EDC. However, reduced growth occurred only at the highest concentration in their exposure series. It may not be useful to measure growth for all treatments. Because growth is a sublethal endpoint, its use has sometimes been restricted to treatments for which survival was not significantly less than that in control treatments (Hunt et al., 1997). Hunt et al. (1997) observed that individuals that survive high toxicant concentrations may often be larger than average. The effect of contaminant exposure on growth may be related to test organism age in addition to contaminant concentration. McKenney (1986) found that older (9 to16d) juvenile mysids showed significantly reduced growth at a lower fenthion concentration than that found for younger (0 to 9d) juveniles. Reduced growth in mysids possibly results from a transfer of energy from growth mechanisms as an organism attempts to counteract stress (McKenney, 1985; 1989; McKenney and Matthews, 1990; Section 6.3 below). Reduced growth has strong implications for reproductive success in mysids as several studies (e.g., Mees et al., 1994; Turpen et al., 1994) have shown that fecundity is directly related to female body size.

6.1.2 Morphology

Changes in morphology resulting from exposure to contaminants have been documented for many taxa, including arthropods. For example, chironomid (midge) larvae, which are commonly used in toxicological testing, can develop mouthpart deformities when exposed to chemicals that affect endocrine systems (Meregalli et al. 2001). The deformities result from physiological disruption during molting. Meregalli et al. (2001) found that sublethal concentrations of 4-n-nonylphenol, a known endocrine disruptor, caused mouthpart deformities in *Chironomus riparius*. Despite the similarities in molting physiology between insects and crustaceans, it does not appear that morphology has been widely considered as an appropriate and measurable endpoint in mysid toxicological studies. A laboratory study showed that exposure to cadmium resulted in the development of abnormal genitalia in male *A. bahia* and malformed carapaces in males and females (Gentile et al. 1982). The time to the first appearance of the abnormalities was positively related to cadmium concentration. One study based on field-collected data found that four populations of *N. integer* contained many individuals with abnormal telson morphology (Mees et al. 1997). Such abnormalities could arise through

physiological perturbations that occur during molting, and they could provide a quantifiable measure of disruption in endocrine-related functions.

The determination of the degree of fluctuating asymmetry (FA) found among mysids could provide a quantifiable, repeatable measure of morphological perturbations resulting from exposure to possible EDCs. FA is the asymmetric development of a normally symmetrical bilateral structure in which there is no tendency for one side of the structure to have a larger value than the other (Palmer and Strobeck 1986; Palmer 1994; Leung et al. 2000). FA is thought to arise because of environmental or genetic stress during development (Clarke 1993; Leung and Forbes 1996) and may result from a shift in metabolic energy from systems that maintain developmental stability to those that help organisms compensate for increased stress (Sommer 1996). Measurement of FA, once appropriate characters have been determined, is relatively easy and requires only use of a microscope. Characters that have been measured are readily observable and include midge larvae teeth (*Chironomus*, Clarke 1993), copepod body spines (*Tisbe*, Clarke,1992), and shrimp antennae (*Palaemon*, Clarke,1993). However, Leung et al. (2000) mentioned that the measurement of single characters does not always reliably indicate environmental stress, and they suggested that an approach involving the use of composite indices of FA could increase the probability of detecting environmental stress. Although some studies of FA have been done on crustaceans (e.g., Clarke 1992, 1993), none has focused on mysids. Before FA could be used to detect mysid developmental abnormalities resulting from exposure to EDCs, preliminary studies examining several suites of potential characters would have to be performed.

6.1.3 Behavior

The disruption of mysid swimming behavior is one endpoint that has recently been investigated as potentially informative in documenting sublethal exposure to contaminants. In a series of studies, Roast et al. (1998, 2000a, 2000b, 2001) determined that sublethal concentrations of cadmium and chlorpyrifos (an organophosphorus pesticide) significantly altered the swimming behavior of *N. integer*. Perturbations included decreased ability to swim against a current, an increase in general activity with no improvement in ability to swim against the current, and a reduced tendency to maintain a position near the bottom. These changes in behavior could have important ecological impacts to the animals by causing them to be moved to an unfavorable habitat or by increasing their susceptibility to predation, thus indirectly resulting in a lethal response to contaminants at sublethal concentrations. However, if the disruption of swimming behavior is to be used as an endpoint in endocrine disruption studies, it should be applied with caution. Roast et al. speculated that the disruption of swimming that they observed was probably a nervous system effect related to interference with cholinergic pathways.

Reduced feeding has been noted as a sublethal response to exposure to some contaminants. For example, Nimmo et al. (1981) noted, but did not quantify, reduced mysid feeding in response to exposure to some pesticides. Although difficult to accurately quantify, reduced feeding no doubt is an important sublethal effect of contaminant exposure that may be

expressed in many of the life cycle parameters described below. Reduced feeding could lead to reduced growth, reduced time to maturity, and reduced egg production, among other factors.

6.2 MEASURES OF REPRODUCTIVE PERFORMANCE

There are several measures of reproductive performance that can be used to assess sublethal response. For example, sexual maturity, the time to first brood release, the time required for egg development, fecundity, and alterations in reproductive characteristics in populations have all been used as endpoints.

6.2.1 Sexual Maturity

Khan et al. (1992) asserted that sexual maturity, which they described as the presence of gonads or a brood pouch, is a feasible endpoint for reproductive tests, because gonad maturation is essentially the first step toward reproductive output. Maturity allows for the measurement of effects to both males and females. Khan et al. used a dissection microscope to examine live mysids for the presence of gonads at the test termination. They quantified maturity as the ratio of the number of sexually mature mysids to the number of surviving mysids in each test replicate, and demonstrated that maturity was a sensitive endpoint for tests of 96-h and 7-day duration that were initiated with 8-day-old mysids. Others used the time required for mysids to reach sexual maturity as a test endpoint (e.g., Gentile et al. 1983; Lussier et al. 1985). Gentile et al. (1983) used the development of testes or the presence of eggs in the oviduct to determine when sexual maturity was reached in male and female mysids, respectively. They reported that exposure to high levels of mercury significantly lengthened the time required for mysids to reach maturity, which then was expressed as delay in the appearance of eggs in the brood pouch and the release of young.

6.2.2 Time to First Brood Release

In uncontaminated systems, the length of time to the release of the first brood is primarily related to environmental temperature, with some influence by salinity and an interaction between the two factors (McKenney 1996). McKenney (1996) determined that the shortest time to release of the first brood for A. bahia was about 16 days at a temperature of 28°C and a salinity of 28‰. The time to release of the first brood increases with decreasing temperature and salinity. Because this parameter is measured as the number of days from hatching of the mysids used in the test until they release their first brood, it can represent the expression of more than one factor, including the length of time it takes a mysid to reach sexual maturity and the time required for eggs to develop in the brood pouch before being released. Most contaminant effects are likely to lengthen the time to release of the first brood. Lussier et al. (1985) found that several metals (mercury, zinc, nickel) significantly increased the time to first brood release, whereas others (e.g., cadmium, copper, silver) did not. Gentile et al. (1983) found that very high levels of mercury caused mysids to abort eggs that had been deposited in the brood pouch.

6.2.3 Egg Development Time

Egg development time is measured as the number of days between the first appearance of eggs in the brood pouch and the first release of juveniles. Gentile et al. (1983) found that mercury did not significantly affect the brood duration, although several other reproductive parameters were affected.

6.2.4 Brood Size (Fecundity)

Brood size can be measured as the number of eggs per brood (Khan et al. 1992) or as the number of young produced, expressed either as total young per female or as young produced per available female reproductive day (Gentile et al. 1982; Lussier et al. 1985). The latter measure is used to normalize differences in the number of females available per test concentration (Gentile et al. 1982). The number of available female reproductive days is calculated by multiplying the number of mature females by the number of days survived. McKenney (1996) showed that the number of eggs in the first brood was related to salinity and temperature, and that the largest number of eggs was produced at a temperature of 25°C and 31‰ salinity. Because it is an important measure of reproductive success, any reduction in brood size can be interpreted as an indication of reproductive toxicity (Khan et al. 1992). However, brood size is also directly related to female size. Therefore, reduced fecundity in response to exposure to EDCs needs to be carefully evaluated to distinguish direct interruption of reproductive processes from a simple reduction in growth. Khan et al. (1992) also stated that the use of fecundity without supporting parameters to indicate reproductive impairment is not advisable, because fecundity is labor-intensive to determine, requires trained personnel, and ignores toxic effects on males. The most likely effect of contaminants is a reduction in fecundity (Hollister et al. 1980; Lussier et al. 1985), which in some cases is the only response to contaminant exposure (Lussier et al. 1999). Contaminant exposure can also result in the abortion of broods (Gentile et al. 1983). Lussier et al. (1999) reported, but could not explain, a seasonal difference in fecundity: more eggs were produced in the fall than in winter or summer. This phenomenon should be considered when comparing tests conducted at different times of the year. Lussier et al. (1999) concluded that fecundity was nonetheless a sensitive and useful endpoint.

6.2.5 Intersexuality

Exposure to EDCs can result in profound alterations in the reproductive characteristics of populations, expressed as physiological or morphological changes in individuals. For example, the most commonly reported phenomenon is a condition, pseudohermaphroditism, in which female molluscs develop male reproductive structures in response to exposure to tributyltin (LeBlanc et al. 1999). Among the Crustacea, cases in which individuals showed intersexuality have been reported for several different taxa (see references cited in LeBlanc et al. 1999). Mees et al. (1997) reported intersexuality in natural populations of *N. integer* collected in northern Europe. However, a link between such phenomena in crustaceans and EDCs has not been established (LeBlanc et al. 1999). In some cases, abnormal sex ratios could be the result of EDC exposure, as has been seen in populations of copepods (Moore and Stevenson 1991, 1994).

6.3 BIOCHEMICAL MEASURES

Five biochemical measures are explored below as possible endpoints for EDC-exposure tests: metabolic disruption, steroid metabolism, vitellogenin induction, and the levels of cytochrome P450 enzymes and of blood glucose.

6.3.1 Metabolic Disruption (O:N ratios)

Many of the perturbations expressed in the parameters described in Sections 6.1 and 6.2 could be related to changes in energy pathways resulting from chronic exposure to contaminants. McKenney (1985, 1989) and McKenney et al. (1991) showed that exposure to contaminants caused increased respiration rates in juvenile mysids, often with as little as 24-h exposure. The increased general metabolic demands related to contaminant exposure reduced growth by decreasing the amount of energy available to produce new somatic tissues. Increased metabolic demands caused by exposure to contaminants could also impair mysid reproductive capability. Young mysids typically use high-energy lipids to meet metabolic demands, but change to metabolize proteins as they mature, thereby leaving more lipid material available for the production of gametes (McKenney 1989). Increased metabolic demands caused by exposure to contaminants is met by greater lipid metabolism, which reduces the lipids available to meet reproductive needs (McKenney 1985, 1989). These changes in metabolic substrate usage can be measured by monitoring the oxygen:nitrogen (O:N) ratio of test organisms. The O:N ratio indicates the relationship between the amount of oxygen consumed by an organism to the amount of nitrogen excreted, and shows the relative role protein catabolism plays in the organism's energy budget (Carr et al. 1985; McKenney 1985). Mysids showed a change toward lipid metabolism after only 4 days of exposure to high concentrations of cadmium (Carr et al. 1985). McKenney (1985) demonstrated that mysids showed increased metabolic demands after 4 days of exposure to thiobencarb, an herbicide, and found high O:N ratios among maturing mysids exposed to the compound, indicating a shift to lipid metabolism that would have reduced the lipids available for gamete production. Both studies concluded that changes the O:N ratio measured among test mysids was a sensitive indicator that could provide for the relatively early detection of reproductive impacts by contaminants.

6.3.2 Steroid Metabolism

Although the role estrogens play in crustacean reproduction is not known (Baldwin et al. 1995), these steroids are important in other invertebrate groups. Because of the likelihood that crustaceans could be exposed to environmental estrogens such as dichlorodiphenyl trichloroethane (DDT), polychlorinated biphenyls (PCBs), and nonylphenols (nonionic surfactants), there is the potential for these compounds to disrupt steroid metabolism. Baldwin et al. (1995, 1997, 1998) studied the effects of three environmental estrogens, diethylstilbestrol (DES), 4-nonylphenol (4NP), and nonylphenol polyethoxylate (NPPE), on the steroid metabolism of the freshwater daphnid, *Daphnia magna*. Their work focused on the disruption of

the metabolic elimination of testosterone after short- and long-term exposure to the test compounds and sought to determine whether such an analysis could be used as an early indication of reproductive impairment. They measured differences in the glucose conjugation, sulfate conjugation and hydroxylated and reduced/dehydrogenated metabolites of ^{14}C-labeled testosterone in daphnids exposed to sublethal concentrations of the test compounds. They found that the different compounds had different effects on testosterone metabolism. For example, DES increased glucose conjugation, but did not affect sulfate conjugation, whereas 4NP reduced both of these elimination processes. In their two earlier studies (Baldwin et al. 1995, 1997), Baldwin's group proposed that changes in testosterone metabolism could provide an early indication of potential reproductive toxicity after sublethal exposure to suspected EDCs. However, in their 1998 paper, Baldwin et al. studied NNPG, a nonionic surfactant that degrades to nonylphenol, and did not find significant disruption of steroid metabolism after short-term exposure. They did report some effects after chronic exposure and postulated that those could have resulted from the degradation of NPPE to NP. Therefore, they cautioned that use of short-term exposures as an early warning indicator might underestimate chronic effects resulting from bioaccumulation and bioactivation of the test compounds. Recently, Verslycke et al. (2002) studied testosterone metabolism in *Neomysis integer*. Significantly, they detected testosterone in male and female mysids. They also found an anabolic steroid, β-boldenone—the first known occurrence of the compound in an invertebrate, in mysids exposed to testosterone added to the test medium. A vertebrate estrogen, 17β-estradiol, was not detected. ·

6.3.3 Vitellogenin

Depledge (unpublished, cited in Depledge and Billinghurst 1999) found that exposure to 4-n-nonylphenol induced the production of vitellogenin in decapods. Vitellogenin production is most likely controlled by ecdysteroids, the hormones primarily involved in molting, although in some crustacean groups this is likely not the case (Subramoniam 2000).

6.3.4 Cytochrome P450 Enzymes

Cytochrome P450 enzymes (CYPs) are commonly occurring proteins that are involved in the metabolism (i.e., detoxification) of many exogenous and endogenous compounds (Snyder 2000; Snyder and Mulder 2001). Snyder and Mulder (2001) measured CYP45 levels, a family of P450 proteins found in the lobster *Homarus americanus*, and thought they must be involved in the molting cycle, in response to exposure to the pesticide heptachlor, a known EDC. They found that peak levels of ecdysteroid hormones, and accordingly, molting, occurred later in heptachlor-exposed larvae than in control larvae, indicating that heptachlor disrupts steroid molting hormone metabolism. They also found CYP45 levels in lobster larvae exposed to heptachlor on Days 1, 2, or 3 after hatching to be 15 times higher than they were in those exposed to control solutions. Levels of CYP45 typically peaked 1 to 2 days after exposure, then decreased. Snyder and Mulder suggested that it could be a useful early biomarker of exposure to EDCs, because it showed a dramatic and rapid increase in levels after exposure to heptachlor. In their testosterone metabolism study, Verslycke et al. (2002) showed that *Neomysis integer* has many P450 enzymes that comprise its complex steroid hydroxylation system. They suggested

that changes in P450 activity could be used as a biomarker indicating exposure of this mysid to EDCs.

6.3.5 Blood Glucose Levels

Levels of glucose in crustacean blood is regulated by a hormone, crustacean hyperglycemic hormone (CHH), that is produced in the sinus gland (Fingerman et al. 1998). Release of CHH increases blood glucose levels. Some exogenous compounds have been shown to affect the levels of glucose in the bloodstreams of several crustacean taxa, probably by stimulating (e.g., naphthalene) or inhibiting (e.g., cadmium) CHH synthesis (Fingerman et al. 1998). Measurement of changes in blood glucose levels in mysids exposed to potential EDCs could be indicative of hormonal perturbation other than that associated directly with reproduction or molting.

7.0 RESPONSE TO ECDYSTEROID AGONISTS AND ANTAGONISTS

Concern has often been expressed in recent years about the disruption of endocrine systems in aquatic organisms by the action of organic and inorganic contaminants (e.g., Snyder and Mulder 2001; Depledge and Billinghurst 1999; Fingerman 1997). In a review by Hutchinson et al. (1999a), it was suggested that based on estimated figures, the concentration of EDCs expected to be introduced to the United States' aquatic environment could be as high as 2.16 ng/L for 17 α-ethynylestradiol-derived contraceptives, and 41.5 ng/L for conjugated estrogens used in hormone-replacement therapy. Although there has been considerable research conducted on the health of fish exposed to EDCs, there is little information available for crustaceans (Baldwin et al. 2001; Hutchinson et al. 1999a, Hutchinson et al. 1999b).

The endocrine and reproductive effects of EDCs are to mimic the effects of natural hormones, to antagonize the effects of hormones, to alter the pattern of synthesis and metabolism of hormones, and to modify hormone receptor levels (Depledge and Billinghurst 1999). The ability of some environmental contaminants to bind to steroid hormone receptors as agonists or antagonists in a recognized mechanism of toxicity to endocrine-related processes has been documented (LeBlanc and McLachlan 1998).

7.1 POSSIBLE GENDER DIFFERENCES IN RESPONSE TO ECDYSTEROIDS

The literature is vague with respect to gender differences from exposure to ecdysteroids. Cuzin-Roudy and Saleuddin (1989) discussed possible differences in effects to male and female mysids, *Siriella armata*. This study showed that secondary vitellogenesis starts at the beginning of the molt cycle for this organism, when ecdysteroid levels are low. There is a striking difference between males and females at this point: in females, ecdysteroid levels were 10 times higher than those in males, but the response of the epidermis for molt preparation was the same. Females also had much higher levels of 20-E, ecdysone, and high polarity products, which are probably linked to the storage of ecdysteroids in oocytes during secondary vitellogenesis. Embryonic and post-embryonic development occur in the marsupium of the females. Juveniles

are released shortly before ecdysis, after which the adult female lays a new batch of eggs in the marsupium. A secondary vitellogenic cycle starts for a new batch of oocytes on the second day of the female molt cycle. Secondary vitellogenesis is strictly linked to the molt cycle. During development, gonads and gonoducts differentiate before the appearance of secondary sexual characteristics (Cuzin-Roudy and Saleuddin 1989).

Crustaceans are in general fast-growing and slow-breeding organisms. Integration between molting and reproduction is a physiological necessity in females. Ecdysteroid, the chief hormone in molting, is thought to be involved with control of female reproductive activities. However, this is controversial. Investigations using amphipods have shown that levels of vitellogenin fluctuate with hemolymph ecdysteroid levels (see for example Cuzin-Roudy and Saleuddin 1989, Depledge and Billinghurst 1999).

In crustacean females, sequestered ecdysteroids may be passed on to the eggs for possible elimination and to function as morphogenetic hormones partaking in the control of embryogenesis and early development. The ovary in many crustaceans accumulates ecdysteroid for possible use during embryogenesis (Subramoniam 2000). Molting and reproduction are more evident in the female because vitellogenesis is the central event of the female reproductive cycle along with secretion of a new cuticle during molting. Hormones play a role in the nutritive supply for molting and vitellogenesis. The ovaries eliminate ecdysteroids by forming ecdysonic acid as a necessary way to eliminate ecdysteroid in the eggs and embryos. They also form conjugates as a means of elimination. In embryos, there are concentrations of the three ecdysteroids–ecdysone, 20-E, and PoA–and their conjugates. There are fluctuations in embryonic ecdysteroids, as evidenced by one shrimp species, *Sicyonia ingentis,* in which the eggs after spawning contain low levels of ecdysteroid. The levels then rise through development, probably by the synthesis of this hormone by the embryo's Y-organ. The endogenous accumulation of ecdysteroid within the ovary is also known to function in the induction of meiotic maturation of the oocyte (Subramoniam 2000).

In one experiment, Subramoniam (2000) removed eggs from the pleopods of the freshwater prawn, *Macrobrachium nobilii.* The release of eggs quickened the next molting and reproductive cycle. In another experiment, Subramoniam (2000) found that although the ovarian cycle begins during the intermolt stage, vitellogenesis (serum levels) progresses into the next premolt stage. Premolt starts with the release of the larvae, and the next spawning occurs after ecdysis. Among penaeid shrimp, free spawning occurs during the premolt stage, followed by ecdysis (Subramoniam 2000).

In summary, detailed studies of crustacean response to ecdysteroids are lacking. Future studies that address sequence determination of vitellogenic genes and their hormonal activity could provide interesting insight into the vitellogenic process in this taxonomic group. Genomic and nongenomic effects of ecdysteroid on ovarian maturation is a potential area of work. Synergistic and antagonistic actions of the X-organ sinus-molt and gonad-inhibiting

neuropeptides, and the mandibular organ control over molting and reproduction are other areas requiring further study as a basis for use of crustaceans for EDC testing in the future.

8.0 RESPONSE TO ANDROGENIC AGONISTS AND ANTAGONISTS

Vertebrate-type steroidal androgens have been measured in some crustaceans, but androgen receptors have not been documented. Steroidal androgens can function directly as hormones in ways that do not require receptors, or they can be present as inactive components of steroid metabolic pathways (LeBlanc and McLachlan 1999). There is currently no published research that evaluates the androgenic hormones and their effect specifically on mysids. Administration of testosterone to shrimp has resulted in hypertrophy and hyperplasia of the androgenic gland. The androgenic gland is associated with the testis, and is responsible for the secretion of the androgenic hormone. This hormone is nonsteroidal and is responsible for masculinization. Testosterone administered to shrimp and crab results in the increase in testis size and in the conversion from ovaries to testes in females (LeBlanc and McLachlan 1999). These results and the recent discovery of endogenous testosterone in *Neomysis integer* (Verslycke et al. 2002) suggested that studies designed to measure androgenic effects in mysids could be conducted.

8.1 ENDPOINT SENSITIVITY

Vertebrate-type steroid hormones are found in the ovaries, testis, mandibular organ, and hemolymph of crustaceans. Many of these steroid hormones exhibit fluctuations during gonadal development, suggesting a role in reproduction of crustaceans. The androgenic gland lies on the vas deferens and is the likely source of the androgenic hormone responsible for the development of the male characters and the production of gametes (Cuzin-Roudy and Saleuddin 1989).

In the shrimp, *Penaeus monodon*, 17 β-estradiol and progesterone in free and conjugated forms increase in the ovary during vitellogenesis (Fairs et al. 1990). Metabolic precursors such as pregnenolone and dehydroepiandrosterone also increase and show a peak during the major vitellogenic stages, suggesting a pathway in crustaceans that is similar to that in vertebrates. Fairs et al. (1990) also reported that 17 β-estradiol and progesterone levels in the hemolymph showed fluctuations resembling that of serum vitellogenin levels during ovarian maturation. Estrogen could possibly control the stimulation of yolk synthesis, whereas the progesterone could control the prophasic meiotic maturation, causing germinal vesicle breakdown in the post vitellogenic oocytes. Exogenous injections of steroidal hormones induced vitellogenesis in the prawn, *P. japonicus*. In a study of a marine shrimp, *P. semisulcatus*, it has been shown that both the vitellogenin synthesis in the hepatopancreas and vitellin synthesis on the oocytes are coded by one gene (Subramoniam 2000).

The identification of physiological targets of EDC in invertebrates is the approach taken by LeBlanc and McLachlan (1998). One example is that diethylstilbestrol and endosulfan have been shown to inhibit molting in immature daphnids, but to have no effect on the mature

animals' fecundity. These effects may indicate that chemicals that are estrogenic to vertebrates could affect molting and reproduction in crustaceans, interfering with the proper function of the ecdysone receptor. In a study designed to examine antiandrogens, *Daphnia magna* was exposed to the compound cyproterone acetate, to determine whether it interferes with the androgen receptor as it does with vertebrates (LeBlanc and McLachlan 1998). The results showed an impairment to growth. The exposed organisms were smaller, and there was a reduction in number of offspring. The latter was most likely due to the smaller size of the organisms, which would not have been able to accommodate a more normal number of brood in the pouch. The effects of steroidal androgens and chemicals that cause metabolic androgenization are consistent with interference to the delivery or packaging of nutrients into the developing eggs. Ecdysteroids, juvenoids, progestogen, and crustacean androgens have all been shown to influence vitellogenin or lipid production in arthropods. Androgens may interfere with one or more of the hormonally regulated processes that provide nutrients to embryos.

Baldwin (1997, 1998) conducted a series of experiments using the daphnid. During one experiment, the daphnid was exposed to 4NP, which resulted in changes in rates of elimination of testosterone and a corresponding decrease in glucose-conjugated testosterone, and an increase in the rate of production of various androgenic derivatives of testosterone. This is called metabolic androgenization, which is found to reduce fecundity of exposed daphnids associated with developmental abnormalities and high mortality of offspring. Results from a separate experiment with exposure to NP revealed no significant evidence of changes in steroid elimination processes, except at the highest concentration, which reduced elimination of glucose and sulfate conjugates and increased elimination of oxido-reduced derivatives. Effects were seen at sublethal levels for 4NP and approaching acute levels for nonylphenol. It has not yet been determined whether there is an androgen receptor in crustaceans; therefore, more studies are needed to determine the functional role of steroidal androgens.

It is possible that endogenous androgens may be precursors to other hormones, and that large doses of exogenously added androgens could elicit activity through other receptors. In crustaceans, testosterone is converted to androstenedione at various rates (LeBlanc and McLachlan 1999). Future studies may reveal that the conversion is affected by age, reproductive state, or photoperiod. It is possible that alteration in testosterone metabolism could serve as a biomarker, because effects are observed at concentrations less than those eliciting reproductive response.

8.2 GENDER DIFFERENCES

Currently, there is no documented research that discerns gender differences in mysids as a result of androgenic-type hormone response. Detailed mechanistic and anatomical studies would need to be conducted on mysids to ascertain whether differences in gender relative to EDCs can be observed.

In the 1980s, the condition of imposex (the imposition of male sex organs including a penis and vas deferens) was observed with increasing frequency on marine gastropods exposed

to tributyltin (TBT) (Depledge and Billinghurst 1999). The mode of action of TBT giving rise to imposex is currently under investigation. Female snails exposed to TBT have elevated testosterone in the hemolymph, and injections of TBT into females induced penis formation (Depledge and Billinghurst 1999). Lee (1991) thought that many of the observed effects in molluscs are related to enzymes involved in TBT metabolism. Inhibition of a cytochrome P450-dependent aromatase (which normally converts 17-β-estradiol to testosterone) could result in the accumulation of testosterone, which would otherwise be metabolized.

Studies of the shrimp, *Palaemon serratus*, showed that eyestalk ablation resulted in rapid maturation of the ovaries (reviewed by Fingerman et al. 1998). It was later shown that this effect is caused by the sinus gland containing a gonad-inhibiting hormone (GIH). This system is present in male crustaceans as well, and eyestalk ablation to induce gonadal maturation is a common practice on shrimp farms worldwide (Fingerman et al. 1998). The presence of gonad-stimulating hormones (GSH) was demonstrated in decapod crustaceans. In female crustaceans, the GIH and GSH acted directly on ovaries, which then secreted the ovarian hormone. Ovaries are a source of ovarian hormone, which induces the development of secondary female sexual characteristics. In male crustaceans, GIH and GSH acted on the androgenic gland. Two experiments were conducted to determine the role of the androgen gland using *Macrobrachium rosenbergii*. When the androgen gland was removed, the male became feminized, and when the androgen gland was implanted into a female, the female became masculinized (Fingerman et al. 1998).

In their review, Fingerman et al. (1998) reported that parasitism of crustaceans by rhizocephalans induced castration. The castration of the males often involved additional impairment to testicular function by modification of the secondary sexual characteristics, causing the males to take on female appearance. For example, the narrow male abdomen of crabs became wider, resembling that of a female. Several authors, such as Fingerman et al. (1998), have reported that in male shore crabs, *Carcinus maenas*, spermatogenesis occurred nonetheless in the testes of specimens found with feminized abdomens.

9.0 RESPONSE TO OTHER HORMONAL DISTURBANCES

In their review paper, Fingerman et al. (1998) described other hormonal responses and disturbances in crustaceans, such as color-changing hormones, retinal pigment hormones, pericardial hormones, and blood glucose hormones. Each of these will be briefly described below, relative to crustaceans in general. Specific hormone disturbances to mysids as well as endpoint sensitivity and gender differences await further study.

The sinus gland is the storage and release site for color-change hormones, among others. The sinus gland is located proximal to the eye and lies next to the large hemolymph sinus (Fingerman 1997). For sessile crustaceans, the sinus gland is located in the head close to the optic centers. Investigators believe that 90% of axonal terminals that compose the sinus gland belong to neurons whose cell bodies lie in the medulla terminalis X-organ. Therefore, the

medulla terminalis X-organ sinus-gland complex is similar to the vertebrate hypothalamo-neurohypophyseal complex (Fingerman 1997).

Color change is affected by cells called chromatophores, which are located in the integument. They are responsible for color change through their dispersion and aggregation. In an early experiment, the hemolymph of a dark prawn specimen was transferred to a pale one (Fingerman 1997). When this organism was kept on a white background, it turned dark. The researcher then cut through the exoskeleton to sever any peripheral nerves that should innervate the chromatophores to determine whether color change was related to the endocrine system or to the nervous system. The incision had no effect on color change. Histological examination failed to show any innvervation of the chromatophores, which led to the conclusion that color changes of this prawn are due to hemolymph-borne pigment concentrating substances.

For a variety of decapod crustaceans, chromatophores that cause integumentary color changes are controlled by antagonistically acting pigment-dispersing and pigment-concentrating neurohormones. For example, in the fiddler crab, *Uca pugilator,* the neurohormone, 5-HT-serotonin triggers the release of red pigment-dispersing hormone, but has no effect on the black chromatophores (Fingerman 1997). The black chromatophores are triggered by norepinephrine, which releases a black pigment-dispersing hormone (BPDH). Studies have shown that the eyestalks of *U. pugilator* contained four times as much BPDH as did the control after exposure to naphthalene, due to naphthalene's inhibition of norepinephrine release. The opposite mechanism was observed for cadmium (Fingerman 1997). The eyestalks of control organisms contained three times more BPDH than did the cadmium-exposed crabs, which indicated that cadmium inhibited the synthesis of BPDH.

Retinal tissue contains pigments that control the amount of light striking the rhabdom (the photosensitive part of each ommatidium that compose the compound eye) through changes in position. Three types of retinal pigments have been categorized: the distal, proximal, and reflecting (Fingerman 1997). Migration of the distal pigment is controlled by the light-adapting hormone (LAH) and the dark-adapting hormone (DAH). Most studies of retinal pigments use the distal pigment, because techniques for its use are noninvasive. Several studies were conducted using *Palaemonetes vulgaris*. In one experiment conducted by Fingerman in 1959, this species was kept under constant illumination and then injected with extracts of eyestalks or sinus glands (described in Fingerman 1997). Because there is an initial light-adapted response followed by a dark-adapted response, Fingerman was able to induce a dark-adapting response from an organism kept under constant illumination, suggesting that eyestalks contain both LAH and DAH.

The sinus gland contains the source of CHH, which causes elevation of blood glucose levels for crustaceans. CHH is similar in structure to MIH. It has been determined that MIH and CHH also show similar activity (Fingerman 1997) Future studies should address the specific roles of these hormones in mysids and other crustaceans. Exposure of the prawn, *M. kistnensis*, and several species of crabs to cadmium caused hyperglycemia. Similarly, exposure of *U. pugilator* to naphthalene caused hyperglycemia, although the mode of action is apparently

different for the two compounds. Cadmium inhibits CHH synthesis, whereas naphthalene stimulates CHH synthesis; 5-HT apparently triggers release of CHH (Fingerman 1997).

Pericardial organs lie in the venous sinus that surrounds the heart, and the axon terminals could be part of the neuroendocrine system that releases hormones affecting the heart. Experiments showed that the hearts of three species, *Cancer pagurus, Homarus vulgaris, and Squilla mantis,* responded to pericardial organ extracts with increase in both frequency and amplitude of the heart beat. Efforts to identify substances in the pericardial organs have revealed 5-HT, dopamine, and octopamine (Fingerman 1997).

10.0 CANDIDATE PROTOCOLS

10.1 ASTM E1191 STANDARD GUIDE FOR CONDUCTING LIFE CYCLE TOXICITY TESTS WITH SALTWATER MYSIDS (ASTM 1997)

American Society for Testing and Materials (ASTM) Method E1191 (ASTM 1997) offers detailed specifications and information for conducting mysid life cycle tests. However, some of the specifications are either vague, or are designed to provide considerable latitude in practice, which can lead to some inconsistency among laboratories, particularly when interlaboratory comparisons of results must be made. For example, guidance regarding the required number of replicates allows each testing laboratory to assign the number of replicates per test, thereby determining the desired level of detectable difference between test and control treatments, and the power of detecting those differences. Also, some of the testing conditions have an allowance for modification to suit the capabilities of particular laboratories to conduct the testing. For example, only general specifications regarding the test chamber size and volume are provided, allowing laboratories some flexibility in choosing the final test apparatus. The protocol recommends the use of a relatively large test chamber that is subdivided into several replicate compartments. This design aspect may not be appropriate for all testing laboratories, especially for those at which the use of individual replicate containers is standard. Some flexibility in design requirements is desirable, but it should be tempered so that interlaboratory comparisons are not sacrificed.

Although the specifications listed in ASTM E1191 are primarily directed to *A. bahia* tests, they are for the most part also directly applicable to two other species, *A. bigelowi* and *A. almyra*. The protocol can also generally be applied to other mysid species (e.g., *Holmesimysis costata*), but may need to be modified to better meet the ecological requirements of the species tested. Various recommendations made in the protocol are supported by relevant literature citations. A summary of the test conditions recommended by this protocol is presented in Table 10-1

10.2   OPPTS TEST GUIDELINE 850.1350 MYSID CHRONIC TOXICITY TEST (EPA 1996)

The EPA Office of Prevention, Pesticides and Toxic Substances (OPPTS) led the development of several protocols that provide guidelines for conducting tests of toxic substances to generate data for the EPA's use. The OPPTS Test Guideline 850.1350 (EPA 1996) provides general guidelines for conducting a mysid chronic toxicity test. It also primarily addresses the requirements for testing with *A. bahia*. Overall, it offers summary-level guidance, but it is not specific in its description of several protocol items. For example, it does not provide a recommended test container size or test volume. Although this approach offers some degree of flexibility to testing laboratories, it increases the likelihood that interlaboratory tests may be difficult to compare because of differences in the application of the protocol. It does not provide literature-based support for the recommendations. A summary of the test conditions recommended by OPPTS Test Guideline 850.1350 is presented in Table 10-1.

10.3   OTHER PROTOCOLS

Specific guidance for conducting short-term toxicity tests with species other than *A. bahia* has been published. It is possible that these protocols can be modified to allow longer life cycle testing.

Holmesimysis costata.— Chapman et al. (1995) described a 7-day test protocol designed to measure growth and survival in tests using the west coast mysid species, *H. costata*. In addition to recommended test conditions, guidance in culturing the animals and analyzing the data are presented. The protocol describes the ecological and culture requirements for *H. costata;* this information could be used to modify the ASTM and OPPTS protocols described above to allow longer life cycle testing. The test protocol was evaluated by means of a series of intra- and interlaboratory comparisons (Hunt et al. 1997), which concluded that this test had sufficient sensitivity and precision to make it useful in testing possible contaminant impacts. A summary of the test conditions recommend by this protocol is presented in Table 10-2.

Mysidopsis intii.— A short-term toxicity test protocol for a west coast species, *M. intii*, was developed with support from the EPA (Langdon et al. 1996). The protocol concisely describes the test conditions required to conduct a 7-day toxicity test to measure survival and growth of this species. Initial test development was performed using zinc sulfate as the toxicant. The test protocol was evaluated by means of an interlaboratory comparison that employed sodium dodecyl sulfate as the toxicant (Harmon and Langdon 1996). Harmon and Langdon (1996) also compared the *M. intii* test with those using *A. bahia* and *H. costata*, and reported that its sensitivity was equal to that of the *A. bahia* test, but that it was lower than that of the *H. costata* test. A summary of the test conditions recommended by this protocol is presented in Table 10-2.

41

Table 10-1. Recommended Mysid Life Cycle Toxicity Test Conditions

	ASTM E1191 (ASTM 1997)	OPPTS 850.1350 (EPA 1996)
Test Species:	Americamysis bahia A. bigelowi A. almyra	Americamysis bahia
Holding Conditions:	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 3°C/12 h salinity at <3‰/24 h) 76-L aquaria Flow through or recirculating system 14 h light:10 h dark, or 16 h light: 8 h dark, with 15–30 min transition period Gentle aeration Feed excess≤ 24 h old Artemia; 150/mysid/day; may supplement with algae or other food	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 1°C/24 h; salinity at <5%/24 h) Flow through or recirculating system 14 h light:10 h dark, with 15–30 min transition period Aeration if needed
Test Setup:		
Test organism age:	≤24 h	≤24 h
Duration:	≥7 day after median first brood release in controls	28 day
Test Material:	Reagent grade or better	NS[a]
Endpoint(s):	Survival, growth, reproduction	Survival, growth, young produced
Number of Treatments:	≥5 plus control (add solvent control if necessary)	≥5 plus control (add solvent control if necessary)
Concentration Series:	Test concentrations should bracket the highest concentration at which there is not an unacceptable effect; each concentration should be at least 50% of the next highest concentration	5 or more concentrations chosen in geometric ratio between 1.5 and 2.0.
Dilution Water: Solvent:	Natural or reconstituted seawater acceptable to saltwater mysids; uniform quality during test; should not affect test outcome Must allow satisfactory survival, growth, and reproduction If solvent used, ≤0.1 mL/L concentration.	Natural (>20-μm-filtered) or artificial seawater If solvent used, ≤0.1 mL/L concentration
Flow Conditions:	Flow through	Flow through
Delivery System: Flow Rate: Calibration limit: Calibration/Check:	Proportional diluter >5 volume additions/24 h (must be capable of 10 additions/24 h) <10%/chamber/time Prior to test; visual check twice daily	Proportional diluter 5 × chamber volume/24 h <10%/chamber/time. Prior to test; twice daily

Table 10-1. Recommended Mysid Life Cycle Toxicity Test Conditions (Contd)

	ASTM E1191 (ASTM 1997)	OPPTS 850.1350 (EPA 1996)
Number of Replicates:	Variable, estimated according to expected variation, desired detection limit, and selected power.	5+ (minimum 40 mysids/treatment)
Test Chamber:	e.g., 300 mm x 450 mm x 150 mm deep with adequate compartments (to provide 30 cm²/mysid).	Volume NS; materials must minimize sorption of test chemicals; loosely covered
Test Volume:	Solution depth ≥100 mm (in above specified chamber)	NS
Number of organisms/rep:	NS (recommends 1 male-female pair/ compartment, but can't determine gender for~12 d)	8 (maximum)
Other Setup Notes:	NS	NS
Test Conditions:		
Light:	NS	NS
Photoperiod:	14 h light:10 h dark, or 16 h light:8 h dark, with 15–30 min transition period	14 h light:10 h dark with 15–30 min transition period
Temperature:	27°C (for *A. bahia*); ± 3°C individual measurements; ± 1°C time-weighted average; <2°C difference between any two jars measured concurrently.	25°C ± 2°C
pH:	6.6–8.2	NS
Dissolved Oxygen: Aeration:	A concentration between 60-100% of saturation is best. yes	60-105% saturation
Salinity:	15–30‰; variation among treatments should be <5, must be <10 ‰	20‰ ± 3‰
Monitoring:		
Test Concentration	Twice prior to test, at 24 h apart; Measured concentration ≤30% of nominal concentration. During test frequently enough to establish average and variability, at least weekly.	At Day 0, 7, 14, 21, 28; should vary <20% among replicates/concentration
WQ Frequency:	Salinity daily; temperature in one chamber hourly or min/max measured daily; pH at start and end of test and weekly in control, include highest concentration; dissolved oxygen in at least one test chamber at start and end and weekly	Weekly (includes pH)
Observation Frequency:	Daily Count, determine gender, remove dead G1 mysids; count live mysids; record number live females Record day of brood release; count and remove young daily Record abnormal development and aberrant behavior	periodically; record number dead on Day 7, 14, 21, 28

Table 10-1. Recommended Mysid Life Cycle Toxicity Test Conditions (Contd)

	ASTM E1191 (ASTM 1997)	OPPTS 850.1350 (EPA 1996)
Feeding:	Live brine shrimp nauplii at least once daily; may supplement Dead brine shrimp should be removed daily before feeding occurs.	Recommend 48-h-old *Artemia*. Frequency and amount not specified.
Other Monitoring Notes:	Weekly determinations of particulate matter, total organic carbon, and total	NS
Termination Notes:	Count live G1 mysids and determine gender Desirable to measure total body length (anterior tip of carapace to tip of uropod) Obtain dry weight of surviving G1 (males and females separate); remove any brine shrimp present; rinse mysids in deionized water, dry at 60°C for 72–96 h Morphological observations at end of test may be desirable May be desirable to hold G2 mysids for 4+ day longer to observe possible effects	Record number of dead on Day 7, 14, 21, 28 Record number of males & females and measure body length (anterior tip of carapace to tip of uropod) when distinguishable and on Day 28. Count and separate G1 offspring as produced, hold at test concentrations. If possible (i.e., by Day 28), count, determine gender and measure G2 mysids. Record abnormal behavior or morphology.
Test Validity Criteria:	A test is valid if General test requirements are met ≥70% G1 control survival ≥75% G1 control females produce young ≥3 average number of young/female	A test is valid if ≥75% G1 control females produce young ≥3 average number of young/female/day
Range-Finding Test		
Concentration Series number of reps test volume test containers number of animals/rep duration	NS	Widely spaced; e.g., 1, 10, 100mg/L 1 NS NS Minimum 10/concentration NS, allow estimate of test concentrations
Termination Notes:	NS	NS
Test Validity Criteria:	NS	NS
Reference Toxicant:	NS	NS
Concentration Series number of reps test volume test containers number of animals/rep	NS	NS
Termination Notes:	NS	NS
Test Validity Criteria:	NS	NS

a) NS Not specified.

Table 10-2. Recommended Test and Holding Conditions for *Holmesimysis costata* and *Mysidopsis intii*[a]

	Chapman et al. (1995)	Langdon et al. (1996) Harmon and Langdon (1996)
Test Species:	*Holmesimysis costata*	*Mysidopsis intii*
Holding Conditions:	WQ similar to test conditions 4, 1000-L tanks ½ volume changed twice/week <20 mysids/L Feed ≤ 24 h old *Artemia* (5–10/mysid/d); plus 10–20 mg ground Tetramin®/100 mysids/d	WQ similar to test conditions 3-L Pyrex glass beakers 2 L volume; 90% exchange every 2 d 15/beaker Feed: 2-d old enriched *Artemia* + *Tigriopus californicus*
Test Setup:		
Test organism age:	3–4 d post hatch	0 or 2 d growth 6 d reproductive condition 15 d reproductive output
Duration:	7 d	7 d
Test Material:	effluent	NS[a]
Endpoint(s):	Survival; growth	Survival; growth, reproductive condition,
Number of Treatments:	NA[b]	NA
Concentration Series:	Minimum of 5 and 1 control	No guidelines specified (see reference toxicant section)
Dilution Water:	Uncontaminated 1-μm-filtered natural seawater or hypersaline brine prepared from natural seawater	NS
Flow Conditions:	Static renewal	Static renewal
Delivery System:	Manual remove/replace	
Flow Rate:	75% renewal at 48 h and 96 h	90% change every 2 d
Calibration:	NA	NA
Calibration Check:	NA	NA
# Replicates:	5	3
Test Chamber:	1000 mL	1-L Pyrex glass beaker
Test Volume:	200 mL	1 L
#organisms/rep:	5	15
Other Setup Notes:	NS	NS
Test Conditions:		
Light:	10–20 μE/m^2/s (ambient laboratory)	1000 lux
Photoperiod:	16 h light :8 h dark	16 h light :8 h dark
Temperature:	13 °C ± 1 °C (N of Pt. Conception) 15 °C ± 1 °C (S of Pt. Conception)	20 °C ± 2 °C
pH:	NS	8.0 ± 0.3

Table 10-2. Recommended Test and Holding Conditions for *Holmesimysis costata* and *Mysidopsis intii* (Contd)

	Chapman et al. (1995)	Langdon et al. (1996) Harmon and Langdon (1996)
Dissolved Oxygen:	>4.0 mg/L	> 60% saturation (at test conditions)
Aeration:	None unless needed to maintain above limit; then < 100 bubbles/min.	None unless needed to maintain above limit.
Salinity:	34 ‰ ± 2 ‰	34 ‰ ± 2 ‰
Monitoring:		
WQ Frequency:	At beginning and end of exposure in one chamber per treatment; temperature daily in two chambers per environmental control system. (WQ includes pH).	Before and after each water exchange
Observation Frequency:	Daily; count and record number of live mysids; remove dead mysids and excess food	number of live mysids recorded at each water exchange
Feeding:	≤24-h-old *Artemia*	≤24-h-old *Artemia* + *Tigriopus californicus* Varies with mysid age
ration	40/mysid/day (two feedings/day, 20 each)	
Other Monitoring	NS	NS
Termination Notes:	Remove and record dead mysids Count survivors (considered alive if visibly respond to stimulus) Screen surviving mysids to remove from chamber, count, rinse in deionized water, transfer to tared weighing boat, dry at least 24 h at 60 °C	Growth and Survival: Remove and count surviving mysids, place in 10 mL dilution water, and refrigerate at 5° C for 24 h Fix in 5% buffered formaldehyde Measure body length (base of eyestalk to posterior edge of exopod) of 10 individuals + Reproductive Condition: Determine percent females carrying eggs or juveniles in brood sac + Reproductive Output: Determine number of juveniles released per final number of females
Test Validity Criteria:	A test is valid if ≥75% control survival ≥0.40 μg dry wt in control; survival MSD^c<40%; growth MSD <50 μg; NOECs , 100 μg/L with zinc.	A test is valid if ≥85% control survival ≥3.0 mm total body length for controls
Reference Toxicant:	Zinc sulfate	NS (protocol development tested zinc sulfate and sodium dodecyl sulfate)

46

Table 10-2. Recommended Test and Holding Conditions for *Holmesimysis costata* and *Mysidopsis intii* (Contd)

	Chapman et al. (1995)	Langdon et al. (1996) Harmon and Langdon (1996)
Concentration Series number of reps test volume test containers number of animals/rep	0, 10, 18, 32, 56, 100 μg/L total zinc 5 NS NS NS	Zinc sulfate: 0, 37, 50, 70, 101, 230 μg/L SDS: 0, 1.58, 2.51; 3.97, 6.30, 10.00 mg/L
Termination Notes:	NS	NS
Test Validity Criteria:	NS	NS

a) NS Not specified.
b) NA Not applicable.
c) MSD Minimum significant difference.

11.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

In this section, we recommend the test species and the protocol for its use that would be most suitable for EDC-determination studies. Gaps in the current knowledge are evaluated, and necessary supplementary studies are recommended.

The utility of invertebrates in general can be justified on the basis of the ease of culture of many species from different phyla that have relatively short life cycles. Although there is limited documentation to date of invertebrate responses to EDCs, we have reviewed in previous sections the studies that have been conducted primarily in the lab, but also a few that were done *in situ*. The reproductive system of invertebrates appears to be particularly vulnerable to EDCs. Of particular interest in using *aquatic* invertebrate species as test organisms is that the neuroendocrine centers in the eyestalk, brain, and thoracic ganglia produce substances that regulate ion movements in tissues of crustaceans in freshwater and saltwater habitats.

11.1 PREFERRED TEST SPECIES

The preferred mysid species fo use in the testing of potential EDCs is *Americamysis bahia*. The primary reasons for its selection are that it is commercially cultured and readily available year-round, it has been the subject of many toxicity tests, it has a short generation time, and its testing requirements and biology are well known. It can be cultured and maintained easily by testing laboratories. These advantages outweigh the disadvantage that in some situations, EDC testing with this species may mean using a test organism that is not indigenous to the geographic area of interest.

11.2 DESCRIPTION OF THE METHOD

The test is initiated when healthy < 24-h-old mysid juveniles are placed randomly into replicate test chambers. These original juveniles comprise the parent (P) mysid stock for the test. The assay is conducted with at least five toxicant concentration treatments and appropriate control treatments (typically a 0% concentration and a solvent control, if one was used to deliver the toxicant to the test treatments). The highest exposure concentration should be below the single-phase solubility of the chemical in the dilution water–no more than 50% nominal exposure concentration (J.M. Neff, personal communication, January 15, 2002).[2] Toxicant and control treatments are delivered to the mysids in water, which is delivered to the test chambers via a proportional diluter system. The specific exposure duration will vary, but is at least 7 days longer than the median brood-release date by the second brood offspring (F1") in the control treatments. The test plan and approximate timeline are shown conceptually in Figure 11-1. During the test, chambers are examined for mysid mortality, the presence of molted exuviae, the presence of ovigerous females, and the release of young, all of which are recorded. Young from the first brood (= F1') release by the parent stock are held for four days, after which they are counted and measured. The parent stock is allowed to produce a second brood (= F1"), after which the parent mysids are counted and measured. The F1" mysids are exposed to the test materials and maintained until their first brood is released (= F2), after which the F1" mysids are counted and measured. The test is terminated with the release of the F2 mysids, which are counted. Surviving organisms may be analyzed biochemically, as appropriate.

11.2.1 General Procedures and Equipment

The test mysids shall be obtained from a single batch of juveniles obtained from the same brood stock. The brood stock must have been hatched and raised in the testing laboratory or obtained by the laboratory prior to sexual maturity and held at test environmental conditions for at least 14 days. Food during holding must be the same as that used during the test. Animals selected for testing must be <24 h old and must not exhibit abnormal behavior or morphology. Brood stock holding tanks must contain no more than 20 mysids/L and be free of other organisms.

The test generally should consist of five toxicant concentration treatments and appropriate control treatments. The toxicant concentration series should bracket the highest concentration at which there is not an unacceptable effect. Each concentration, except for the control and the highest concentration, should be at least 50% of the next highest concentration.

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[2]It is not possible to estimate the actual exposure concentration–that is, the concentration of bioavailable chemical in true solution–when saturated or nearly saturated solutions are used for the stock solution or for the highest exposure concentrations (J.M. Neff, personal communication, January 5, 2002).
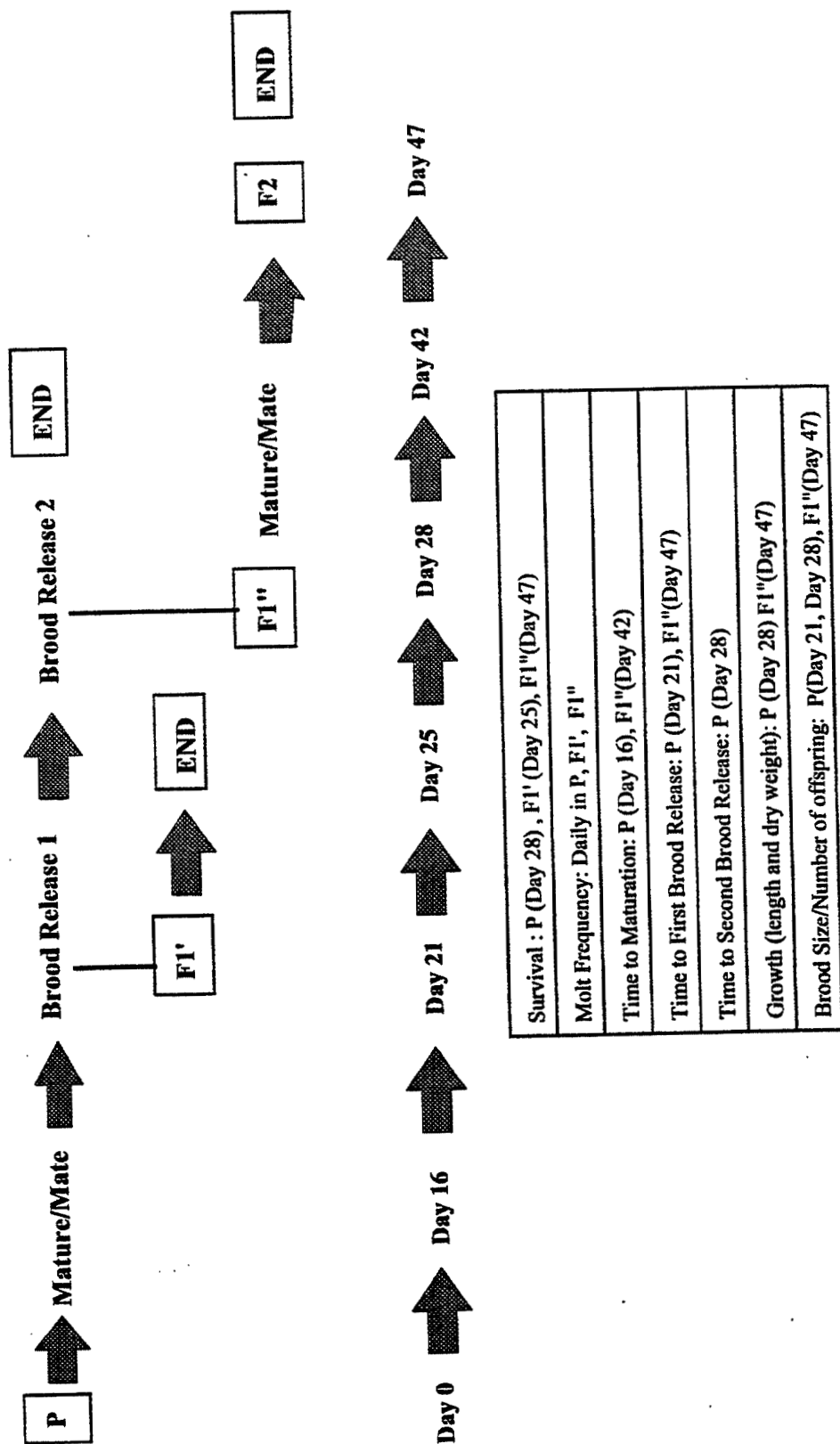
Figure 11-1. Conceptual Diagram of Measurements Endpoints and Estimated Corresponding Timeline for the Mysid Two Generation Test

Timeline elements:

P → Mature/Mate → Brood Release 1 → Brood Release 2

F1' → END

F1'' → Mature/Mate → F2 → END

Day 0, Day 16, Day 21, Day 25, Day 28, Day 42, Day 47

Endpoint
Survival : P (Day 28) , F1' (Day 25), F1''(Day 47)
Molt Frequency: Daily in P, F1', F1''
Time to Maturation: P (Day 16), F1''(Day 42)
Time to First Brood Release: P (Day 21), F1''(Day 47)
Time to Second Brood Release: P (Day 28)
Growth (length and dry weight): P (Day 28) F1''(Day 47)
Brood Size/Number of offspring: P(Day 21, Day 28), F1''(Day 47)

49

The definitive test concentration series can be determined by using known results from toxicity studies or by conducting a range-finding test in which the concentrations tested are widely separated, such as values of 1, 10, and 100 mg/L. The experimental unit for the mysid test is the test chamber and is defined as the smallest unit to which treatments can be independently applied. The suggested test chamber that is used is a 250-mL glass jar that has been modified to permit flow-through conditions. ASTM (1997) and other protocols call for the use of relatively large tanks that are subdivided into compartments. However, the toxicant concentration in such cases is not applied independently to all compartments, which are therefore not considered experimental units or replicates.

The number of replicates used in various protocols and tests ranges from two to eight. Although it may be desirable to determine the number of replicates based on the expected variation, desired detection limit, and selected power, as suggested by ASTM (1997), the number selected will most likely be constrained by the capacity of the diluter system. However, a minimum of four replicates per treatment is recommended.

The requirements for a given test facility should accommodate the use of flow-through or recirculating tanks for both holding and testing. The ideal would be to use proportional diluters with an elevated head box to allow for gravity-fed dilution water into the brood tanks or chemical mixing chambers. A metering system could be used to mix and deliver test concentrations to the appropriate testing chamber. The test chamber must be maintained at a constant temperature using either temperature-controlled water or recirculating water baths. The water and air going into the testing system should be strained of any particulate matter using either strainers or air and water filters capable of filtering material through a 0.20-micron bacterial filter (ASTM 1997; EPA 1996).

Lighting conditions for testing require the use of timers capable of delivering light for a 14-h light and 10-h dark cycle with a 15 to 30 minute transition period. The transition period is important, because mysids may become stressed by instantaneous changes in light. In the natural environment, the normal vertical migration of mysids allows for gradual acclimation to light changes. Under laboratory conditions, instantaneous change in light has been observed to cause jumping or impingement on the sides of the testing container (ASTM 1997).

The test facility should be well ventilated and free of fumes. Laboratory ventilation systems should be checked to ensure that return air from chemistry laboratories or sample handling areas is not circulated to culture or testing rooms, or that air from testing rooms does not contaminate culture rooms. Air pressure differentials between rooms should not result in a net flow of potentially contaminated air to sensitive areas through open or loose-fitting doors. Air used for aeration must be free of oil and fumes. Oil-free air pumps should be used where possible. Filters to remove oil, water, and bacteria are desirable. Particles can be removed from the air using filters such as BALSTON Grade BX (Balston, Inc., Lexington, Massachusetts) or equivalent, and oil and other organic vapors can be removed using activated carbon filters (e.g., BALSTON C-1 filter) or equivalent (EPA 2000). During phases of rearing, holding, and testing, test organisms should be shielded from external disturbances such as rapidly changing light or pedestrian traffic (EPA 2000).

Equipment and supplies that contact stock solutions, sediment, or overlying water should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and supplies that contact sediment or water should be chosen to minimize sorption of test materials from water. Glass, type 316 stainless steel, nylon, and high-density polyethylene, polypropylene, polycarbonate, and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Concrete and high-density plastic containers may be used for holding and culture chambers, and in the water-supply system. These materials should be washed in detergent, acid-rinsed, and soaked in flowing water for a week or more before use. Cast-iron pipe should not be used in water-supply systems, because colloidal iron will be added to the overlying water and strainers will be needed to remove rust particles. Copper, brass, lead, galvanized metal, and natural rubber must not contact overlying water or stock solutions before or during a test. Items made of neoprene rubber and other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect survival, growth, or reproduction of the test organisms (EPA 2000).

For administration of test substance via water, the recommended equipment consists of proportional diluters, metering systems, pumps, or other suitable systems to be used to deliver test substances to the testing chambers. The system that is chosen should permit the mixing of test material with dilution water before its entrance into the testing chambers, and should supply the selected concentrations in a reproducible fashion (ASTM 1997).

The system must be calibrated before use to determine the flow rate into the chambers and the test concentration entering each chamber. It is advisable to allow the test solutions to flow through the system for a time sufficient to allow concentrations of the test concentrations to reach a steady state. Then two sets of samples should be taken at least 24 h apart. The chemical analysis of the concentrations should verify that the test concentrations have reached a steady state before organisms are placed into testing chambers. The measured test concentrations should be no more than 30% of estimated or nominal concentrations. The 24-h flow rate into the test chambers should be at least five volume additions. The flow rate through any two chambers should not differ by more than 10%. The general operation of the test delivery system should be checked twice per day, usually morning and afternoon (ASTM 1997; EPA 1996).

11.2.2 Test Validity

The test acceptability criteria generally follow those listed in EPA (1996), with some guidance from the ASTM (1997) procedure. Basic principles of experimental design must be followed. All test chambers must be identical, treatments must be randomly assigned to test chamber locations, all appropriate control treatments must be included, and individual mysids must be allocated randomly or impartially into the test chambers. Tests conditions (e.g., water quality) must be within the specified guidelines. Control treatment survival for first-generation mysids must be at least 70%. At least 75% of the first generation females in the control treatments must produce young, and the average number of young produced per female must be at least three per day.

11.3   ENDPOINTS: APPROPRIATENESS AND PREFERRED METHODS FOR QUANTIFICATION

11.3.1   Reproductive and Development Endpoints

Because potential EDCs may elicit more than one response and the responses may vary with the chemical tested, several endpoints are included in the testing program. Many of the adverse responses to exposure to toxicants that are typically measured in toxicity testing programs (e.g., survival, growth, reproductive biology) are important in determining whether or not a potential EDC could cause significant impacts to wild populations. However, all of these responses might not be attributable to the disruption of endocrine processes. Therefore, to conclude that a particular toxicant is an endocrine disruptor, it is also necessary to establish that exposure to toxicants has affected endocrine systems.

Endpoints that can indicate possible impacts to populations that should be included in a toxicity test are described below.

Survival is recorded as the proportion of individuals still living at the termination of the appropriate phase of the test. Mysids are considered dead if they are opaque white in color, immobile (especially regarding respiratory and feeding appendages), and do not respond to gentle prodding. Missing animals should be considered dead. Survival is determined for P, F1', and F1" mysids.

Molting frequency is regulated by the endocrine system and may be directly impacted by EDCs. Disruption of molting affects other measured parameters such as reproduction and growth. Molting frequency is determined by daily collection and recording of the number of molted exuviae per chamber. These data are used to calculate the number of molts per mysid per day. Molting frequency is determined for P, F1', and F1" mysids.

Growth is a sublethal developmental endpoint that can be reduced by exposure to EDCs or other stressors, and is therefore appropriate to include. The inclusion of growth as an endpoint may help distinguish between the responses. Growth is measured as the difference in the length and/or dry weight of test organisms before and after exposure to test and control treatments. The length measurement should be made prior to the dry weight determination. Mysids should be relaxed by chilling, or by using some other method that does not affect subsequent weight measurements, prior to being measured. Mysid length is measured (to the nearest 0.1 mm) for each individual as the distance along the midline of the body from the tip of the carapace to the tip of the exopod (excluding the terminal setae). After obtaining the length measurements, dry weight should be measured by rinsing all surviving mysids carefully in deionized water to remove salt and drying them at 60°C until constant weight is reached (usually ~72–96 h). Weight measurements should be made to the nearest 0.01 mg (as suggested in EPA 2000). Constant weight is reached when successive measurements differ by less than 0.1 mg. Growth is measured by replicate for P and F1" mysids.

Reproduction measurements including sexual maturity, time to first brood, brood size, and offspring produced (total and average per female), are described in detail as follows:

- *Time to sexual maturity* is calculated as the duration, in days, of the interval between the initiation of the test (P mysids), or the release of the second P-mysid brood (F1" mysids), and the appearance of structures defining maturity. Maturity is defined as the appearance of oostegites in the female and by the development of testes in the male. Some authors have used the presence of eggs in the oviduct as the indication of maturity in female mysids. Time to sexual maturity is determined for P and F1" mysids.

- The *time to first brood* is the time, measured in days, from hatching of the test organisms to the release of the first brood of young. Time to first brood is determined for P and F1" mysids. Time to second brood is determined for P mysids.

- Brood size is the number of eggs per brood produced by a single female. It could also be characterized as the number of offspring produced by a single female. Brood size is determined for P and F1" mysids.

- The *total number of offspring produced* is determined as the total number of young produced per replicate over the course of the experiment. Because the number of females per replicate may change during the test (primarily because of mortality in higher test concentrations), the metric can be normalized to the number of females available per test concentration by calculating the number of available female reproductive days. The number of available female reproductive days is determined by multiplying the number of mature females by the number of days survived. The number of offspring produced can also be expressed as the average number of young per female per treatment. The number of offspring produced is determined for P and F1" mysids.

11.3.2 Biochemical Endpoints

Several metabolic measurements have been strongly associated with alterations in endocrine-related processes after exposure to sublethal concentrations of toxicants. However, further research is needed before these endpoints can be considered for inclusion.

Metabolic disruption occurs as stress induces changes in the substrates used in metabolism. It is determined by measuring the O:N ratios of the test organisms. This ratio indicates the relationship between the amount of oxygen consumed by an organism and the amount of nitrogen excreted, and shows the relative role protein catabolism plays in the organism's energy budget (Carr et al. 1985; McKenney 1985). Changes the O:N ratio measured among test mysids is a sensitive indicator that could provide for the relatively early detection of reproductive impacts by contaminants.

Disruption in steroid metabolism by EDCs can be determined by studying metabolic elimination of testosterone by mysids after exposure to the test compounds. Difference in metabolic byproducts such as glucose conjugation, sulfate conjugation and hydroxylated and reduced/dehydrogenated metabolites of ^{14}C-labeled testosterone in mysids exposed to sublethal concentrations of the test compounds can be measured. Different EDCs can affect testosterone

metabolism in varying ways. For example, DES increased glucose conjugation, but did not affect sulfate conjugation, whereas 4NP reduced both of these elimination processes (Baldwin et al. 1995, 1997, 1998). Therefore, tests should not rely on measurements on only one byproduct. Recent work with mysids (Verslycke et al. 2002) indicated that these measurements will be useful in studies of the effects on potential EDCs on that group.

Vitellogenin induction in crustaceans is probably controlled by ecdysteroids. However, whether or not this is true for mysids in not known. Differences in vitellogenin production among treated and nontreated mysids could provide evidence of endocrine system disruption and should be explored during prevalidation studies.

Cytochrome P450 enzyme levels may be affected by exposure of the crustaceans to EDCs. Measurements of differences in CYP levels between treated and nontreated mysids could provide direct evidence of disruption of steroid molting hormone levels.

Blood glucose levels in crustaceans are regulated by crustacean hyperglycemic hormone produced in the sinus gland. Changes in blood glucose levels in mysids exposed to potential EDCs could indicate disruption of hormonal activity other than that associated with molting or reproduction.

11.4 EXPOSURE PROTOCOL

Because there is no validated two-generation mysid toxicity test, the exposure protocol selected for the EDC mysid testing is one modified primarily from the OPPTS protocol (EPA 1996), but one that also includes suggestions found in ASTM (1997) and Lussier et al. (1988), among other procedures. The suggested protocol and notes about some of the conditions are presented in Table 11-1.

11.5 RESULTS AND REPORTING

11.5.1 Interpretation of Results

Two test-derived calculations are the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC). The LOEC is the lowest concentration of the toxicant to which the mysids were exposed that adversely affected the test animals. The NOEC is the highest concentration to which the mysids were exposed that did not adversely affect the test animals. For an observed response, each value is determined as the concentration that differs statistically from the response measured for mysids in the control treatments. Because each value must be one of the concentrations used in the testing, inferences based on either must not be used as definitive indication of the effects concentration for a particular toxicant.

Table 11-1. **Mysid Two Generation Toxicity Test Conditions Recommended for Conducting Tests of Potential Endocrine Disrupting Chemicals**

	Recommended Protocol	Notes
Test Species:	*Americamysis bahia* Mysids used in test must originate from laboratory cultures	Restricted species to *A. bahia*; see text
Holding Conditions:	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 1 °C/24 h; salinity at ≤ 5%/24 h)	EPA (1996); ASTM (1997). Removed ASTM restriction on holding tank size to allow more flexibility.
	Source for holding/culture water must be the same as for dilution water	Feeding ration from ASTM (1997).
	Holding facility should have same background colors and lighting intensities as testing areas	
	Facility should be well ventilated and free of fumes that could affect test organisms	
	Flow through or recirculating system; latter with ability to filter water as necessary	
	14 h light:10 h dark, or 16 h light:8 h dark, with 15–30 min transition period	
	Gentle aeration	
	Feed excess *Artemia*; # 150/mysid/d; may supplement with algae or other food	
Definitive Test		
Test organism age:	≤24 h	EPA (1996)
Duration:	≤7d after median first brood release in F1" controls	Time estimate is based on expectations for controls; treatments may be delayed in responses so test should extend beyond control response. Expected duration could be up to 55 d
Test Material:	Reagent grade chemical or better	
Reproductive and Development Endpoints:	Survival (P, F1', F1") Molt Frequency (P, F1', F1") Time to Maturation (P, F1") Time to First Brood Release (P, F1") Time to Second Brood Release (P) Growth: length and dry weight (P, F1', F1") Brood Size/Number of Offspring (P, F1")	See Section 6.0 for descriptions of endpoints.
Number of Treatments:	Minimum 5 concentrations, plus control (add solvent control if necessary)	EPA (1996)
Concentration Series:	Use geometric series in which the ratio between concentrations is from 1.5 to 2.0.	EPA (1996). Use range-finding test (described below) to determine definitive test concentrations. The lowest test concentration should not result in an observable effect; the highest should elicit a statistically significant effect.

Table 11-1. Mysid Two Generation Toxicity Test Conditions Recommended for Conducting Tests of Potential Endocrine Disrupting Chemicals (Contd)

	Recommended Protocol	Notes
Dilution Water:	Natural or artificial seawater acceptable to saltwater mysids; uniform quality during test; should not affect test outcome; same source as culture water If natural, must use 0.2 micron-filtered Must allow satisfactory survival, growth, and reproduction	EPA (1996)
Solvent:	If solvent used, ≤0.1 mL/L concentration.	
Flow Conditions:	Flow through	EPA (1996); ASTM (1997)
Delivery System: Flow Rate: Calibration limit: Calibration/Check:	Proportional diluter 5 - chamber volume/24 h <10% variation in flow/chamber/time Prior to test; check twice daily	EPA (1996)
# Replicates:	4 (minimum)	The number of replicates used in various protocols and tests ranges from 2 to 8. EPA (1996) suggests at least 5. Although it may be desirable to determine the number of replicates based on the expected variation, desired detection limit, and selected power as suggested by ASTM (1997), the number selected may be constrained by the capacity of the diluter apparatus.
Test Chamber:	250 mL (minimum) glass jar (appropriate to test volume used)	EPA (1996) does not specify. ASTM (1997) and some other procedures use large tanks into which screened compartments are placed, e.g., 300 mm H 450 mm H 150 mm deep with adequate number of internal compartments (to provide 30 cm^2/mysid). Some procedures use 500–600 mL glass jars.
Cover:		Test chamber should be covered and have a screened overflow port.
Test Volume:	100 mL (minimum)	
Organisms/replicate	1 male:female pair per compartment; minimum 3 compartments per replicate	EPA (1996) specifies a maximum of 8; ASTM recommends 1 male-female pair/compartment; other test procedure use 5–40 mysids per replicate
Test Conditions:		
	NS •	
Photoperiod:	14 h light:10 h dark, with 15–30 min transition period	EPA (1996)
Temperature:	27 °C ± 1 °C	EPA (1996) suggests 25 °C ± 2 °C; higher temperature improves growth and reproduction (McKenney 1996; ASTM 1997; Lussier et al. 1999)
pH:	6.6–8.2	ASTM (1997); NS in EPA (1996)

Table 11-1. Mysid Two Generation Toxicity Test Conditions Recommended for Conducting Tests of Potential Endocrine Disrupting Chemicals (Contd)

	Recommended Protocol	Notes
Dissolved Oxygen: Aeration:	Concentration between 60–100% saturation at test conditions Yes, gentle	EPA (1996)
Salinity:	25 ‰ ± 2 ‰	EPA (1996) suggests 20 ‰ ± 3 ‰; higher salinity improves growth and reproduction (Lussier et al. 1988; McKenney 1996; ASTM 1997)
Monitoring:		
Test Concentration	Day 0, 7, 14, 21, 28, etc., and last day of test	EPA (1996)
WQ Frequency:	Salinity, temperature daily in one replicate chamber per concentration pH at start and end of test and weekly in control, include highest concentration DO in at least one test chamber at test initiation and termination and weekly	EPA (1996) suggests weekly for all, may not be adequate for salinity and temperature
Observation Frequency:	Daily Count, determine gender, remove dead mysids; count live mysids; record # live females Count and remove molted exuviae Record day of first brood release (P, F1); Record day of second brood release (P) Count and remove young daily Record abnormal development and aberrant behavior	
Feeding:	Live brine shrimp nauplii twice daily Dead brine shrimp should be removed daily before feeding occurs.	NS in EPA (1996)
Other Monitoring Notes:		ASTM (1997) suggests that weekly determinations of particulate matter, TOC, and total dissolved gasses are desirable
Data Collection/ Termination Notes:	P: Record time to maturation (d); time of Brood 1 release; # of B1 offspring; time of Brood 2 release; ; # of B2 offspring / Termination: count survivors, weigh and measure length F1': Hold 4 d / Termination: count survivors, weigh and measure length F1": Record time to maturation (d); time of Brood 1 release; # of B1 offspring / Termination: count survivors, weigh and measure length ALL: Record cast exuviae per container per	Data collection points and test terminations vary according to generation. See text for details.

Table 11-1. Mysid Two Generation Toxicity Test Conditions Recommended for Conducting Tests of Potential Endocrine Disrupting Chemicals (Contd)

	Recommended Protocol	Notes
Test Validity Criteria:	A test is valid if General test requirements, including water quality requirements, are met ≥75% parental control females produce young ≥3 average # young/female/d	EPA (1996)
	Range-Finding Test	
Concentration Series	Widely spaced; e.g., 1, 10, 100 mg/L	EPA (1996)
# reps	1	
test volume	400 mL	NS in EPA (1996)
test containers	500-mL wide mouth jar	NS in EPA (1996)
# animals/rep	Minimum 10/concentration	
duration	48–96 h	NS in EPA (1996); long enough to allow estimation of test concentrations
Termination Notes:		NS in EPA (1996)
Test Validity Criteria:		NS in EPA (1996)
	Reference Toxicant Test	
Toxicant	Copper sulfate	NS in EPA (1996)
Concentration Series	0, 150, 200, 300, 400 Tg/L Cu 3 3	
test volume	400 mL	
test containers	500-mL wide mouth jar	
# animals/rep	10	
Termination Notes:	Count surviving mysids	
Test Validity Criteria:	90% survival in controls; data sufficient to calculate LC_{50}	

ᵃ NS, not specified.

These indicate the concentration of a given toxicant that would cause an effect, cause a reduction in a nonquantal measurement (e.g., growth), or cause death, respectively, in a given percentage of the test population. For example, the LC_{50} is the concentration that caused death in 50% of the test population. Calculation of point estimates should always include the calculation of the 95% confidence limits. Point estimates may be calculated by several methods including the Graphical Method, the Probit Method, the Trimmed Spearman-Karber Method, or the Linear Interpretation Method. Section 5.2.1 described the route of administration of chemicals for water. Although this DRP mainly discusses the mysid test in terms of water exposure, there are documented applications of sediment testing using mysids. It is important to note that if a sediment specific protocol has not been developed to examine the effect on EDCs to crustaceans, then mysids may be used to evaluate EDCs in this matrix.

11.5.2 Reporting Requirements

The report should contain all pertinent information that is suggestive or predictive of chronic toxicity. The record of the results of an acceptable test should include the following information either directly or by referencing available documents:

- Name of test and investigator(s), name and location of laboratory, and dates of start and end of test.

- If applicable, source of test water or sediment, and method for collection, handling, shipping, storage, and disposal of sediment.

- Source of test material, lot number if applicable, composition (identities and concentrations of major ingredients and impurities if known), known chemical and physical properties, and the identity and concentration(s) of any solvent used.

- Source of the dilution water, its chemical characteristics, and a description of any pretreatment, and results of any demonstration of the ability of an organism to survive or grow in the water.

- Source, history, and age of test organisms; source, history, and age of brood stock, culture procedures; and source and date of collection of test organisms, scientific name, name of person who identified or cultured the organisms and the taxonomic key used, age or life stage, means and ranges of weight or length, observed diseases or unusual appearance, treatments used, and holding procedures.

- Source and composition of brine shrimp; concentrations of test material and other contaminants in the brine shrimp; procedure used to prepare food; and feeding methods, frequency and ration.

- Description of the experimental design and test chambers, the depth and volume of solution in the chamber, the number of mysids, the number of replicates, the loading, the lighting, and test substance delivery system and the flow rate as volume additions per 24-h period.

- Methods used for physical and chemical characterization of water or sediment and the measured concentrations of test substances in test chambers. Information should include the schedule for obtaining samples for analysis, and the results of the analysis of test concentrations.

- Range including minimum, maximum and average of measured water quality parameters (dissolved oxygen, salinity, temperature, and pH).

- A table of the biological data for each test chamber for each treatment, including the control(s), in sufficient detail to allow independent statistical analysis, including such measurements as time to sexual maturity (P, F1"); length of time to first brood (P,

F1"); length of time to second brood (P), the average and respective confidence intervals for dry weight and/or body length of males and females at sexual maturity and at test termination (P, F1"); cumulative number of young produced per female (P, F1"), cumulative number of dead adults on Days 7, 14, 21, 28, 35, 42, 49, etc. until the final termination of the test (P, F1"), and number of surviving F' mysids 4 days post release. If available, prior to test termination, effects on second-generation mysids (number of males and females, number of molted exuviae collected per mysid per day, growth, and cumulative mortality) should be recorded.

- Definition(s) of the effects used to calculate LC_{50} or EC_{50}, biological endpoints for tests, and a summary of general observations of other effects.

- A table of data on survival, growth, and reproduction of mysids in each test chamber, treatment, and control in sufficient detail to allow for statistical analyses.

- Methods used for and results of the statistical analyses of data.

- Summary of general observations on other effects or symptoms.

- Anything unusual about the test, any deviation from these procedures, and any other relevant information.

- Published reports should contain enough information to clearly identify the methods used and the quality of the results.

11.6 SIGNIFICANT DATA GAPS FOR PROTOCOL OPTIMIZATION

Most of the testing with mysids has focused on short-term, first-generations studies. Specifics regarding carrying these tests for longer duration and incorporating a second generation need to be evaluated. For example, which F1" cohorts should be used for producing F2s, the length of time for observing the parent and offspring generations, and the appropriate performance criteria for the validation of a successful test? These types of questions could be addressed during pre-validation studies.

In addition, the necessity of a two-generation exposure duration should be assessed. Is the production of F2 offspring a more sensitive indicator than production of F1 offspring? Likewise, is the production of F2 offspring alone adequate or would survival or sex ratio or other endpoints be more sensitive still? Pre-validation studies are needed to determine the most cost-efficient and sensitive test duration and endpoints to be included in an optimized protocol suitable for progressing through full validation.

The goal of designing and conducting detailed chronic toxicity tests with mysids would be to determine whether specific endpoint responses can be determined for different classes of compounds that affect ecdysteroid, androgen, or other hormonal cycles. Table 11-2 is an example of the type of information that might be obtained from such studies.

Table 11-2. Measurement of Effects of Three Classes of Hormones

| | O:N Ratio | Survival | Growth | Reproduction | | | | Steroid Metabolism |
				Time to first Brood	Clutch Size	Offspring Viability	Sex Ratio	
Ecdysteroid Agonist								
Ecdysteroid Antagonist								
Androgen Agonist								
Androgen Antagonist								
Other								

11.7 RESEARCH NEEDS

Because growth and development endpoints could be affected directly or indirectly by a variety of stress factors, such as environmental and biological, as well as chemical, it can be difficult to attribute the effects to a specific causal agent or mechanism with certainty. Therefore, biochemical studies must be included in the experimental regime to help verify that the observed endpoints resulted from disturbance of hormone systems. However, prevalidation studies about these biochemical metrics as they relate to mysids still needs to be done. For example, detailed mechanistic studies should be conducted using mysids to determine whether observed endpoint effects are caused by EDCs. Such studies could include receptor blocking/binding studies, endpoint response to specific classes of compounds, and enzyme studies, for example.

Detection of alterations in *steroid metabolism* could provide evidence supporting the impact of a potential EDC on mysid endocrine systems. Studies of testosterone metabolism in daphnid crustaceans showed that differences in glucose conjugation, sulfate conjugation, and hydroxylated, and reduced/dehydrogenated metabolites of ^{14}C-labeled testosterone exposed to test compounds versus control treatments provided evidence for disruption of endocrine processes. The general procedure (e.g., Baldwin et al. 1998) for measuring testosterone metabolites after exposure to test treatments is to place mysids in small containers that contain solutions having the same concentration of test substance to which they were exposed and to which radio-labeled testosterone has been added. Mysids are then homogenized on ice in distilled water, centrifuged, and the supernatant collected to estimate the soluble protein concentration. The soluble protein values are used to normalize rates of testosterone metabolism among treatments. Details of the methods that should be applied to the mysid toxicity tests need to be developed.

- The general procedure for quantifying *glucose- and sulfate-conjugation* metabolites is by hydrolyzation with β-glucosidase (glucose conjugates) or sulfatase (sulfate conjugates) followed by thin-layer chromatography (e.g., Baldwin et al. 1998).

- The general procedure for quantifying *hydroxylated and oxido-reduced/dehydrogenated* testosterone metabolites is by ethyl acetate extraction, and steam evaporation, followed by thin layer chromatography and quantification via scintillation spectography and comparison to known standards (e.g., Baldwin et al. 1998).

Changes in *cytochrome P450 enzymes*, which function in the detoxification of many exogenous and endogenous compounds, may be associated with disruption of the hormonally regulated molting process and are therefore appropriate to measure in EDC studies. The general procedure for determining levels of cytochrome P450 in mysids involves homogenization of whole animals, centrifugation, and the collection of the resulting supernatant. Quantification of the levels occurs via gel electrophoresis. Snyder and Mulder (2001) described a specific method for determining CYP45 (a family of CP450 enzymes) levels in daphnid crustaceans.

It would be necessary to develop biomarkers for the selected species; promising biomarkers could be the induction of vitellogenesis in males, and the inhibition of aromatase in females (Depledge unpublished, cited in Depledge and Billinghurst 1999). As more evidence for endocrine disruption responses is gathered from experimental research, mechanistic studies would be required to determine the specific ways in which chemicals can disturb hormones. That is, it becomes important to distinguish between endocrine disruption and metabolic toxicity, and to determine which is a primary, and which is a secondary, effect. The final step in the strategy would be to conduct field surveys to detect and confirm that endocrine disruption effects occur *in situ* (Depledge and Billinghurst 1999).

12.0 IMPLEMENTATION CONSIDERATIONS

To implement the recommended protocol, regulatory and other legal requirements must be met, and long-term goals for public health and safety should be kept in mind. Following the general principles put forth by ICCVAM, prevalidation studies should be initiated. None of the biochemical endpoints suggested in the recommended protocol have been through a validation process and none have been routinely used by laboratories. Based on available information, selection of the appropriate biochemical endpoints would be difficult. It is therefore recommended that a prevalidation study be performed that would evaluate the biochemical endpoints as markers for endocrine disruption in mysids.

Validation of the study design through interlaboratory comparisons should be conducted once preferred endpoints have been identified using compounds that span the possible endocrine effects, including strong and weak androgen receptor agonists and antagonists, estrogen receptor agonists and antagonists, and thyroid agonists and antagonists

12.1 ANIMAL WELFARE

Legislation governing the care and use of laboratory animals in the United States is contained in the Animal Welfare Act, passed in 1966 and amended in 1970, 1976, and 1985. The Animal Welfare Act covers all warm-blooded animals except mice, rats, birds, and horses, and other farm animals when they are not used for research. It spells out requirements for veterinary care, adequate food and water, protection from temperature extremes, shelter from outdoor elements, sanitation, and record keeping. Because mysids are cold-blooded, there are no current laws governing the culturing or testing of these organisms. Nationally recognized protocols such as ASTM E1191 (ASTM 1997) and OPPTS 850.1350 (EPA 1996) will be followed for testing. These protocols state that all mysids used in testing must be destroyed at the end of the test using humane methods. One humane method that may be used is placing the mysids into a solution of oxygenated MS 222 (euthanizing agent).

12.2 RECOMMENDED EQUIPMENT AND CAPABILITY

To ensure interlaboratory comparability and the general accessibility of the protocol to a broad number of testing laboratories, the following essential equipment and or capabilities are recommended to properly conduct the mysid chronic test:

- Diluters or other flow-through systems with capability of meeting flow and dilution precision requirements
- Low-intensity dual-channel laser-scanning confocal microscope for anatomical measurements (e.g., male-female characteristics, eyestalk structure)
- Analytical measurement capacity – chemical analysis of test compounds
- Protein testing – electrophoresis, chromatography capability, vitellin-probes such as biodipy
- Culture and maintenance of mysids (laboratories can purchase <24-h-old mysids from commercial suppliers).

12.3 TESTING WITH NON-NATIVE SPECIES

Interest in the occurrence and impacts of introduced marine and estuarine species has increased since the 1980s. Recently, Ruiz et al. (2000) documented that about 300 introduced species (invertebrates and algae) have become established in United States coastal waters. Introduced species are significant stressors to coastal ecosystems (Ruiz et al. 1999), and the damage they cause to coastal ecosystems is well documented (e.g., Grosholz and Ruiz 1995, 1996). Governmental initiatives have been implemented to reduce the likelihood of new introductions (Federal Register 1999). Because the primary species recommended for the EDC testing program is not indigenous to some of the geographic regions where testing may occur, all testing laboratories should take appropriate precautions to reduce the possibility that an accidental introduction to a local ecosystem could occur. All test mysids should be destroyed in an appropriate manner at the completion of each test (ASTM 1997).

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72

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74